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(57) Abstract

A DNA sequence for a human mdr1 gene, which encodes p-glycoprotein, wherein at least one base in a splice region of the DNA encoding p-glycoprotein is changed. Such a mutation prevents truncation of the p-glycoprotein upon expression thereof. There is also provided a method of identifying cells which express the human mdr1 gene in a cell population that has been transfected with an expression vehicle including a human mdr1 gene. The method comprises contacting the cell population with a staining material, such as rhodamine 123, and identifying cells which express the human mdr1 gene based on differentiation in color among the cells of the cell population. This method has allowed identification of retroviral producer clones facilitate mdr gene transfer into primary cells. Repopulating hematopoietic stem cells have been genetically engineered with the human mdr1 gene.

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**IMPROVED EXPRESSION OF HUMAN
MULTIDRUG RESISTANCE GENES AND IMPROVED
SELECTION OF CELLS TRANSDUCE WITH SUCH GENES**

This invention relates to the transduction of cells with human multidrug resistance (mdr) genes and the expression thereof, as well as the selection or identification of cells transduced with such genes. More particularly, this invention relates to the enhanced expression of mdr genes by transduced cells, and an improved method of selecting or identifying cells transduced with mdr genes.

Genes for multidrug resistance such as, for example, mdr1 and mutated forms thereof, encode proteins of a class known as p-glycoprotein, that confer resistance to a range of cytotoxic drugs used in the treatment of human malignancies. The introduction and expression of the mdr1 gene in human cells may serve several useful purposes in the treatment of diseases. For example, the mdr1 gene can function as a dominant selectable marker that will allow for positive selection of cells transduced by an expression vehicle, such as, for example, a retroviral vector, ex vivo or in vivo. Used in this manner, expression of the mdr1 gene in normal human cells may be useful in increasing the proportion of a target cell population that has been transformed by an expression vehicle, such as a retroviral particle. Also, expression of the mdr1 gene in normal human

cells may permit more intensive use of chemotherapeutic drugs in the treatment of cancer. For example, expression of the mdr1 gene in human bone marrow cells could reduce the severity and duration of cytopenias following chemotherapy, which may facilitate dose intensification while reducing morbidity and mortality.

In accordance with an aspect of the present invention, there is provided human mdr1 DNA or RNA which encodes p-glycoprotein or an analogue or fragment of such DNA or RNA which encodes an iso-form of p-glycoprotein which confers multidrug resistance. The DNA or RNA includes at least one splice site, and at least one base in the splice site has been changed to a different base which thereby inactivates the splice site. Preferably, the DNA or RNA is a DNA or RNA sequence for human mdr1 which encodes p-glycoprotein wherein at least one base in a splice site of said DNA encoding p-glycoprotein is changed.

The natural human mdr1 gene contains at least one functional splice donor site and splice acceptor site. This invention provides a modification of the mdr1 gene whereby at least one base in a splice site has been changed to a different base which thereby inactivates the splice site.

The term "human mdr1 gene" as used herein means the wild type human mdr1 gene, which encodes p-glycoprotein, as well as mutated human mdr1 genes which encode what are known as iso-forms of p-glycoprotein. Such mutated genes include a mutation in which the human mdr1 DNA encodes a p-glycoprotein in which the first 23 amino acids at the N-terminal are deleted (Currier, et al., J. Biol. Chem., Vol. 264, pgs. 14376-14381 (1989)); insertional mutation(s) in the human mdr1 DNA in which amino acids have been inserted into ATP binding or utilization sites (Currier, et al; 1989); a complementary human mdr1 DNA sequence (cDNA sequence) in which codon 185, which encodes Gly 185, has been changed from GGT to GTT to encode Val 185. (PCT Application No. WO87/05943; Choi, et al., Cell, Vol. 53, pgs. 519-529 (1988);

Safa, et al., Proc. Nat. Acad. Sci., Vol. 87, pgs. 7225-7229 (1990)); and a chimeric gene encoding a protein having adenosine deaminase added to the N-terminal of p-glycoprotein (Germann, et al., J. Biol. Chem., Vol. 264, pgs. 7418-7424 (1989)).

Although the scope of this aspect of the present invention is not to be limited to any theoretical reasoning, Applicants have found that aberrant splicing of the mdr1 gene may occur within the coding sequence for p-glycoprotein. Such splicing, which uses cryptic splice donor and splice acceptor sites, results in the production of expression vehicles (such as, for example, recombinant retroviruses) that contain a truncated mdr1 gene, which encodes a truncated and non-functional p-glycoprotein.

Applicants have found a cryptic splice donor site having a length of 9 bases from nucleotide 760 to nucleotide 768 of the human mdr1 gene, and which has the following sequence:

CAGGTATGC

A cryptic splice acceptor site was also found, which has a length of 16 bases from nucleotide 2729 to nucleotide 2744 of the human mdr1 gene; and which has the following sequence:

ACATTTTTCCTTCAGG

Applicants have found that, by changing at least one base of the cryptic splice donor and/or acceptor sites, one may suppress aberrant splicing of the mdr1 gene and maximize the transfer of DNA encoding functional p-glycoprotein into desired cells.

In one embodiment, the at least one base which is changed is in a splice donor site. In another embodiment, the at least one base which is changed is in a splice acceptor site. In yet another embodiment, at least one base is changed in a splice donor site and at least one base is changed in a splice acceptor site.

In a preferred embodiment, the at least one base in a splice site which is changed is in the splice donor site and is at least

one of guanine (G) or thymine (T) contained in the splice donor site wherein G and T are adjacent.

In another preferred embodiment, the at least one base in a splice site which is changed is in the splice acceptor site and is at least one of adenine (A) or guanine (G) contained in the splice acceptor site wherein A and G are adjacent.

In a more preferred embodiment, at least one base is changed in the splice donor site wherein the at least one base is at least one of G or T contained in the splice donor site wherein G and T are adjacent; and at least one base is changed in the splice acceptor site wherein the at least one base is at least one of A or G contained in the splice acceptor site wherein A and G are adjacent.

In another embodiment, the at least one base in a splice site is changed such that a codon encoding an amino acid is changed to a different codon encoding the same amino acid. Preferably, the at least one base is the third, or "wobble" base in the codon.

In one embodiment, codon 139, located in the splice donor site, and which encodes Arg, is changed from AGG to AGA, which also encodes Arg. In another embodiment, codon 733, located in the splice acceptor site, and which encodes Gln, is changed from CAG to CAA, which also encodes Gln. It is also contemplated that in yet another embodiment, both codon 139 of the mdr1 gene is changed from AGG to AGA, and codon 733 of the mdr1 gene is changed from CAG to CAA.

In one embodiment, the DNA sequence is further mutated such that at least a portion of the 5' untranslated region of the DNA has been removed.

In another embodiment, the DNA sequence may be further mutated such that at least a portion of the 3' untranslated region of the DNA has been removed. In yet another embodiment, the DNA sequence is further mutated such that at least a portion

of the 5' untranslated region of the DNA and at least a portion of the 3' untranslated region of the DNA have been removed. Although Applicants do not intend to be limited thereby, the DNA of the human mdr1 gene is quite large and contains extended 5' and 3' untranslated regions. Such extended regions may result in the generation of a reduced titer of viral vector particles, such as retroviral vector particles, which may be engineered with such DNA. Thus, removal of at least a portion(s) of the 5' and/or 3' untranslated regions may enable one to insert such DNA into a viral vector, such as a retroviral vector, whereby one generates an increased titer of retroviral particles. Within the scope of the present invention, up to the entire 5' untranslated region and/or up to the entire 3' untranslated region may be removed.

In accordance with another aspect of the present invention, there is provided DNA (or RNA) encoding a protein which provides for multidrug resistance, wherein the DNA includes the following sequence:

CAGGTATGC

At least one base is changed to a different base. Preferably at least one base of the GT doublet is changed.

In accordance with yet another aspect of the present invention, there is provided DNA (or RNA) encoding a protein which provides for multidrug resistance, wherein the DNA includes the following sequence:

ACATTTTTCCTTCAGG

At least one base is changed to a different base. Preferably, at least one base of the AG doublet is changed.

In one embodiment, the DNA encoding a protein which provides for multidrug resistance includes a first sequence.

CAGGTATGC, and a second sequence:

ACATTTTTCCTTCAGG

At least one base in the first sequence, and at least one base in the second sequence is changed to a different base. Preferably, at least one base of the GT doublet of the first sequence is

changed, and at least one base of the AG doublet of the second sequence is changed.

The changed DNA or RNA sequence for a human mdr1 gene, or the changed DNA or RNA encoding a protein which provides for multidrug resistance, of the present invention may be cloned into any of a variety of expression vectors by genetic engineering techniques known to those skilled in the art. Such expression vectors include, but are not limited to, prokaryotic vectors, including bacterial vectors; eukaryotic vectors, such as, for example, yeast vectors and fungal vectors, and viral vectors, such as, but not limited to, retroviral vectors, and non-retroviral vectors such as, but not limited to, adenoviral vectors, adeno-associated viral vectors, and Herpes virus vectors. In one embodiment, the changed DNA (or RNA) is cloned into a retroviral expression vector. It is also contemplated that the changed DNA or RNA may be introduced into cells by means of non-viral systems and non-plasmid-based systems. Such systems include, but are not limited to, the injection of the naked changed DNA or RNA into a desired cell, liposomes which encapsulate such DNA or RNA, and which deliver such DNA or RNA to a cell, and the coupling of the DNA or RNA to a protein or other agent which binds to a cellular receptor.

Retroviral vectors which may be employed include those derived from Moloney Murine Leukemia Virus, Moloney Murine Sarcoma Virus, Rous Sarcoma Virus, Harvey Sarcoma Virus, and Spleen Necrosis Virus. In one embodiment, the retroviral vector may be derived from Moloney Murine Leukemia Virus and is one of the LN series of vectors, as described in Miller, et al., Biotechniques, Vol. 7, pgs. 980-990 (1989). Such vectors have a portion of the packaging signal derived from a mouse sarcoma virus, and a mutated gag initiation codon.

In another embodiment, the retroviral vector may include at least four cloning, or restriction enzyme recognition sites, wherein at least two of the sites have an average frequency of

appearance in eukaryotic genes of less than once in 10,000 base pairs; i.e., the restriction product has an average DNA size of at least 10,000 base pairs. Preferred cloning sites are selected from the group consisting of NotI, SnaBI, SalI, and XhoI. In a preferred embodiment, the retroviral vector includes each of these cloning sites.

When a retroviral vector including such cloning sites is employed, there may also be provided a shuttle cloning vector which includes at least two cloning sites which are compatible with at least two cloning sites selected from the group consisting of NotI, SnaBI, SalI, and XhoI located on the retroviral vector. The shuttle cloning vector also includes at least one desired gene which is capable of being transferred from the shuttle cloning vector to the retroviral vector.

The shuttle cloning vector may be constructed from a basic "backbone" vector or fragment to which are ligated one or more linkers which include cloning or restriction enzyme recognition sites. Included in the cloning sites are the compatible, or complementary cloning sites hereinabove described. Genes and/or promoters having ends corresponding to the restriction sites of the shuttle vector may be ligated into the shuttle vector through techniques known in the art.

The shuttle cloning vector can be employed to amplify DNA sequences in prokaryotic systems. The shuttle cloning vector may be prepared from plasmids generally used in prokaryotic systems and in particular in bacteria. Thus, for example, the shuttle cloning vector may be derived from plasmids such as pBR322; pUC 18; etc.

Examples of retroviral vectors having at least two restriction enzyme recognition sites having an average frequency of appearance in eukaryotic genes of less than once in 10,000 base pairs, and of the shuttle cloning vectors employed in transferring genes to such vectors are further described in PCT Application No. WO91/10728.

The vectors also include one more promoter. Suitable promoters which may be employed include, but are not limited to, the retroviral LTR; the SV40 promoter; and the human cytomegalovirus (CMV) promoter described in Miller, et al., Biotechniques, Vol. 7, No. 9, pgs. 980-990 (1989), or any other promoter (e.g., cellular promoter such as eukaryotic cellular promoters including, but not limited to, the histone, pol III, and β -actin promoters). Other viral promoters which may be employed include, but are not limited to, adenovirus promoters, TK promoters, and B19 parvovirus promoters. The selection of a suitable promoter will be apparent to those skilled in the art from the teachings contained herein.

The vector may also further include a heterologous or foreign gene.

Heterologous or foreign genes which may be placed into the vectors of the present invention include, but are not limited to genes which encode cytokines or cellular growth factors, such as lymphokines, which are growth factors for lymphocytes. Other examples of foreign genes include, but are not limited to, genes encoding soluble CD4, Factor VIII, Factor IX, ADA, the LDL receptor, ApoI, tumor necrosis factors (TNF's) and ApoC.

Suitable promoters which may control the foreign genes include those hereinabove described.

The vector including the DNA of the present invention may be transduced into a suitable packaging cell line. Examples of suitable packaging cell lines include the PA317 cell line and the PE501 cell line (Miller, et al., 1989), and the PAT 2.4 cell line (U.S. application Serial No. 792,281, filed November 14, 1991.) Transduction of the packaging cell line may be accomplished by standard techniques such as electroporation or CaPO_4 precipitation. Vector particles generated from such producer cells may then be employed to generate a producer cell line, and to transduce cells (e.g., eukaryotic cells such as mammalian cells), which may be administered to a host as part of a gene

therapy procedure. Examples of cells which may be transduced include, but are not limited to, primary human cells such as primary human nucleated blood cells (such as leukocytes, and lymphocytes such as TIL cells, T-lymphocytes, and B-lymphocytes), tumor cells, endothelial cells, epithelial cells, keratinocytes, stem cells, bone marrow cells, hepatocytes, connective tissue cells, fibroblasts, mesenchymal cells, mesothelial cells, and parenchymal cells.

Upon transfection or transduction of a packaging cell line with a retroviral vector, it is essential that the producer cell line generate a sufficient titer of vector particles for gene therapy protocols. Selection for growth of such packaging or producer cells in the presence of colchicine has resulted in the isolation of producer clones with viral titers below what is currently adequate for clinical gene therapy protocols.

It is therefore another object of the present invention to provide a method of selecting producer cells which generate an acceptable titer of retroviral particles including a human mdr1 gene, and in particular for generating an adequate titer of retroviral particles suitable for inserting and expressing the mdr1 gene in primate cells, particularly in primate repopulating hematopoietic stem cells.

In accordance with an aspect of the present invention, there is provided a method of identifying cells which overexpress a gene encoding multidrug resistance (preferably such gene is the human mdr1 gene) in a cell population transduced with an expression vehicle including a gene which encodes a protein which provides for multidrug resistance (preferably such gene is the human mdr1 gene). The method comprises contacting the cell population with a staining material, and identifying the cells which overexpress the gene encoding a protein which provides for multidrug resistance (preferably the human mdr1 gene) based on differentiation of color among the cells of the cell population. Such differentiation in color may be based on differences in

color intensity between cells which overexpress the human mdr1 gene and cells which do not overexpress the human mdr1 gene (for example, cells which contain vectors including the human mdr1 gene, whereby such cells overexpress the human mdr1 gene, may be brighter or duller than those cells which do not contain vectors including the human mdr1 gene, whereby such cells which express the human mdr1 gene at normal levels or do not express the gene); or differences in color or shades of color between cells which contain vectors including the human mdr1 gene, and thereby overexpress the human mdr1 gene, and cells which do not contain vectors including the human mdr1 gene, and thereby express the human mdr1 gene at normal levels or do not express the human mdr1 gene. Alternatively, cells which contain vectors including the human mdr1 gene, and thereby overexpress the human mdr1 gene may change color after staining, while cells which do not contain vectors which include the human mdr1 gene do not change color after staining.

The term "overexpress" as used herein, means that the gene encoding multidrug resistance is expressed at a level above that which is expressed by a normal human cell. In normal human cells, the mdr1 gene is expressed at low levels or is not expressed. In cells transduced with an expression vehicle including the mdr1 gene, the expression level of the mdr1 gene is considerably increased, and thus, the mdr1 gene is "overexpressed."

Applicants have found that, by identifying cells which overexpress the human mdr1 gene in a cell population transduced with infectious viral particles which include the human mdr1 gene, in accordance with the method of the present invention, Applicants have been able to isolate clones which produce higher viral titers than clones selected with drugs such as colchicine, for example.

In one embodiment, the staining material is a fluorescent dye. Fluorescent dyes which may be employed include, but are not

limited to, rhodamine 123, 3,3'-diethylthiobarbiturate iodide (DiOC₂), and 3,3'-diethylthiobarbiturate cyanine iodide (DODC iodide).

In one embodiment, the fluorescent dye is rhodamine 123. Rhodamine 123 ordinarily concentrates within the mitochondria of cells, but can be effluxed from the cell by p-glycoprotein. Cells stained with rhodamine 123 that do not express p-glycoprotein or express p-glycoprotein at normal or lower than normal levels appear "bright" by fluorescence microscopy or by FACS analysis, whereas cells that overexpress p-glycoprotein appear "dull," even if small quantities of p-glycoprotein are expressed. (Chaudhary, et al., Cell, Vol. 66, pgs. 85-94 (July 12, 1991)). Thus, producer cells or other cells which have incorporated and express a functional human mdr1 gene may be distinguished from non-transformed cells on the basis of this "dull" staining phenotype.

Such a method may also be employed to determine viral titer of human mdr1 retrovirus producer cell lines. In one embodiment, a defined number of target cells are exposed to serial dilutions of a viral supernatant produced by a defined number of producer cells. After infection (eg., at about 48 hrs. after infection), the cells are stained with rhodamine 123 and allowed to efflux the dye. The proportion of "dull" cells can be visually estimated by fluorescence microscopy, or accurately quantitated by scoring a defined number of individual cells by FACS analysis. Because the proportion of cells overexpressing p-glycoprotein is known, it is possible to calculate accurately the minimum number of infectious viral particles present in a known volume of viral supernatant.

Although it has been demonstrated that retroviral vectors may be used to transfer and express the mdr1 gene in cultured cells (Proc.Nat.Acad.Sci., Vol. 85, pgs. 1595-1596 (1988)); in murine repopulating hematopoietic stem cells (Sorrentino, et al., Science, (in press)), and in murine hematopoietic progenitors in vitro (McLachlin, et al., J.Nat.Canc.Inst., Vol. 82, pgs.

1260-1263 (1990)), there has been no demonstration of transfer and expression of the mdr1 gene in primate cells, and in particular in primate stem cells capable of reconstituting transplant recipients.

Thus, in accordance with an aspect of the present invention, there is provided a primate cell which is genetically engineered with DNA (RNA) which encodes a protein which provides multidrug resistance. The DNA (RNA) may be any DNA (RNA) which encodes a protein which provides multidrug resistance or the changed DNA or RNA sequences which are hereinabove described.

Applicants have discovered that by selecting for cells transduced with a gene encoding multidrug resistance in accordance with the selection method hereinabove described, one is able to obtain producer cells which generate a sufficient titer of infectious viral particles which provide for the transfer and expression of multidrug resistance genes in primate cells, in particular in primate repopulating hematopoietic stem cells.

Primate cells which may be genetically engineered with such DNA (RNA) include, but are not limited to, primate bone marrow cells, preferably enriched primate bone marrow cells, and hematopoietic progenitor (CD34+) cells or hematopoietic stem cells (in particular, primate repopulating hematopoietic stem cells), and human primary cells. The hematopoietic stem cells may also be CD33⁻ and HLA-DR^{low}. (Chaudhary, et al., 1991) The primate cells may be obtained from humans or other primates, such as monkeys, for example.

This aspect of the present invention is particularly applicable to the use of primate repopulating hematopoietic stem cells which are genetically engineered with DNA (RNA) which encodes a protein which provides multidrug resistance. Such stem cells give rise to bone marrow cells and may be administered, for example, to a patient who has undergone chemotherapy and/or radiation treatment in order to regenerate healthy bone marrow

cells in the patient. Thus Applicants have devised a system for identifying producer cells which generate a sufficient titer of viral particles including a gene encoding multidrug resistance, whereby such viral particles may be used to infect primate repopulating hematopoietic stem cells, which may then be administered to a patient in order to regenerate bone marrow in said patient, wherein such regenerated bone marrow is resistant to the toxic effects of chemotherapy.

In one embodiment, bone marrow cells are harvested by needle aspiration from a primate, and purified by positive selection for cells expressing the CD34 antigen. CD34 selected cells are enriched 50-100 fold in clonogenic hematopoietic progenitors and contain all of the repopulating stem cells. Alternatively, the bone marrow cells may be purified in accordance with the procedures described in U.S. Patent No. 5,061,620.

The CD34+ cells are incubated in vitro in the presence of stimulatory hematopoietic growth factors and in the presence of retroviral supernatant containing infectious viral particles which include a multidrug resistance gene. The viral particles are generated from a producer cell line selected in accordance with the selection procedure hereinabove described. After incubation, the cells are transplanted into recipients suitably treated to receive a bone marrow transplant. After transplantation, bone marrow cells, peripheral blood leukocytes, purified granulocytes, and T-lymphocytes are assayed for presence and expression of the introduced mdr1 gene.

The invention will be described with respect to the following examples; however, the scope of the present invention is not intended to be limited thereby.

EXAMPLE 1

A. Construction of plasmid pG1.

Plasmid pG1 was constructed from pLNSX (Palmer et al., Blood, 73:438-445; 1989). The construction strategy for plasmid pG1 is shown in Figure 1. The 1.6 kb EcoRI fragment, containing

the 5' Moloney Sarcoma Virus (MoMuSV) LTR, and the 3.0 kb EcoRI/ClaI fragment, containing the 3' LTR, the bacterial origin of replication and the ampicillin resistance gene, were isolated separately. A linker containing seven unique cloning sites was then used to close the EcoRI/ClaI fragment on itself, thus generating the plasmid pGO. The plasmid pGO was used to generate the vector plasmid pG1 by the insertion of the 1.6 kb EcoRI fragment containing the 5' LTR into the unique EcoRI site of pGO. Thus, pG1 (Figure 3) consists of a retroviral vector backbone composed of a 5' portion derived from MoMuSV, a short portion of gag in which the authentic ATG start codon has been mutated to TAG (Bender et al. 1987), a 54 base pair multiple cloning site (MCS) containing from 5' to 3' the sites EcoRI, NotI, SnaBI, SalI, BamHI, XhoI, HindIII, ApaI, and ClaI, and a 3' portion of MoMuLV from base pairs 7764 to 7813 numbered as described in (Van Beveren et al., Cold Spring Harbor, Vol. 2, pg. 567, 1985). (Figure 2). The MCS was designed to generate a maximum number of unique insertion sites, based on a screen of non-cutting restriction enzymes of the pG1 plasmid, the neo^R gene, the β -galactosidase gene, the hygromycin^R gene, and the SV40 promoter.

B. Construction of pG1MD1.

pMDR2000 (Ueda, et al., PNAS, Vol. 84, pgs. 3004-3008 (May 1987)), which contains an mdr1 cDNA sequence (Figure 4) described in PCT application number WO87/05943, wherein the first 282bp of the untranslated 5' region and the last 23bp of the untranslated 3' region of the cDNA sequence have been removed, was cut with SacI and EcoRI, and was inserted into a multiple cloning site of SacI and EcoRI digested pGEM2 (Figure 5.) (Promega, Madison, WI) to form pGEM2MDR. pGEM2MDR was then cut with SmaI at the 5' end of the multiple cloning site and a SacII linker having a length of 8bp was inserted. At the 3' end of the plasmid, the plasmid was cut with EcoRI and a 12bp XhoI linker was inserted. The resulting plasmid was pMDR2000XS. (Figure 6.)

Plasmid pMDR2000XS was then cut at the 5' end of the cDNA sequence for mdr1 with HhaI, and at the 3' end of the cDNA sequence for mdr1 with HaeII, and a 3,845bp fragment was removed. Such cutting removed an additional 131 bp from the 5' end, and an additional 384 bp from the 3' end of the cDNA. A linker was then added to the 3' sequence to reconstitute the amino acids that were cut off by cutting with HaeII and to add the TGA stop (or termination) codon back into the sequence. The linker has four restriction enzyme sites, HpaI, XhoI, BglII, and ClaI, and has the following sequence:

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5'-A G T G A A C T C T G G T T A A C T C C
      10                               20
A C T C G A G C A C A G A T C T G G A
      30
C A T C G A T A C T C
40                               50

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Bases 12-17 are recognized by HpaI, bases 22-27 are recognized by XhoI, bases 31-36 are recognized by BglII, and bases 41-46 are recognized by ClaI.

pG1 was then cut with SnaBI and ClaI, and the mdr1 fragment containing the added linker was cut with ClaI and inserted into the SnaBI and ClaI digested pG1 to form pG1MD1 (Figure 7.)

C. Changing codons 139, 185, and 733 of the mdr1 gene.

Changing the mdr1 gene in pG1MD1 such that codon 139 is changed from AGG to AGA, codon 185 is changed from GTT to GGT (whereby Val is changed to Gly in the resulting expressed p-glycoprotein), and codon 733 is changed from CAG to CAA may be accomplished by either of the following strategies.

1. Introduction of point mutations into G1MD1 by site-directed mutagenesis.

pG1MD1 is digested with NotI and XhoI, and a NotI-XhoI fragment is cloned into the phagemid pBluescriptSK+ (Stratagene). The resulting subclone is transformed into E. coli strain CJ236 (Biorad). A 5ml culture is grown at 37°C overnight in LB broth

with 200µg/ml ampicillin and 30µg/ml chloramphenicol. 2.5ml of this culture is then added to 25 ml of fresh media with the same antibiotics, and grown at 37 C until the O.D.600 of the culture reaches 0.3. Helper phage R408 (Stratagene) is then added at a multiplicity of infection of 20 to the culture. The culture is then incubated by vigorous shaking for 8 hours. The culture is then harvested by centrifugation at 17,000xg for 15 minutes at 4°C. The supernatant fluid is harvested and spun again at 17,000xg for 15 minutes at 4°C. The supernatant is then harvested and measured for volume.

A 1/4 volume of a solution containing 3.5 M ammonium acetate and 20% polyethylene glycol is then added to the supernatant to precipitate the phage. The solution and supernatant mixture is incubated at room temperature for 15 minutes. The precipitate is then centrifuged at 11,000xg for 15 minutes at 4°C. The resulting pellet is suspended in 400µl TE (10mM Tris, 1mM EDTA) and extracted with phenol/chloroform until the interphase is clear. The DNA (phage/template) is then ethanol precipitated by standard procedures.

2ng of the following antisense oligonucleotides:

(a) 5'-T C T G T A C T G G T C T A T A C G G A T A A T
10 20

A A T G T C - 3'

30 , wherein the TCT sequence (nucleotides 11-13) will provide for a change of codon 139 from AGG to AGA;

(b) 5'-C T A A T T A C T T C C A T A A C C A C T G T T

T T A A C C - 3'

30

, wherein the CCA sequence (nucleotides 11-13) will provide for a change of codon 185 from GTT to GGT (whereby Val 185 is changed to Gly 185); and

(c) 5' - G T A A A A G G A A G T T C C A A A G T G T A
10 20

A A C C G T - 3'

30 , wherein the GTT sequence (nucleotides 12-14) will provide for a change of codon 733 from CAG to CAA, and 200ng of the phage/template DNA are then mixed in 10µl of TE. The mixture is heated to 70°C, and then allowed to cool slowly to room temperature in order to anneal the oligonucleotides to the template. Deoxynucleotide triphosphates are then added to the mixture, followed by T4 DNA polymerase, T4 ligase, 10X synthesis buffer, and water to make the volume 20µl. The mixture is then placed on ice for 5 minutes, then at 25°C for 5 minutes, and then at 37°C for 90 minutes.

At the end of the 90 minute incubation, 90µl of TE is added and 5µl of the resulting mixture is used to transform E. coli strain DH5α.

Mutagenized clones are then identified by colony hybridization with the oligonucleotides (a), (b), and (c) hereinabove described. The identified and changed mdr1 gene is then cut out of pBluescript with NotI and XhoI and cloned into pG1 cut with NotI and XhoI to form pG1MD3.

2. Introduction of Point Mutations into G1MD1 by PCR Based Strategy

The cryptic splice donor site and the Gly to Val point mutation in codon 185 are contained within a NotI to ApaI restriction fragment of pG1MD1. This NotI to ApaI fragment can be excised, purified, and subcloned into a pUC based cloning vector called pUC007 (Sorrentino, et al., Nucleic Acids Research, Vol. 18, No. 9, pgs. 2721-2731 (1990)) that contains a synthetic polylinker multiple cloning site. The vector is prepared by partial digestion of pUC007 with EspI, and complete digestion with ApaI. The insert is prepared by digesting with NotI, blunting the NotI digested end with Klenow, and then digesting with ApaI. Following ligation, the abutment of the blunt EspI site with the blunted NotI site will recreate a NotI site that can be used to excise a NotI to ApaI fragment.

Following subcloning of the NotI to ApaI fragment into pUC007 (to form pMD1NA), the cryptic splice donor site and the codon 185 point mutation will be contained within a unique BstXI to MscI fragment. A strategy of overlapping or "recombinant" PCR to introduce point mutations into this BstXI to MscI fragment is employed. (Higuchi, PCR Protocols: A Guide to Methods and Applications, Innis, et al., eds, San Diego, Academic Press, pgs. 177-183 (1990)). Three sets of PCR primers are made. One set of complementary primers (SD-A and SD-B), which have the following sequences:

SD-A: GGAAGACATGACCAGATATGCCTATTATTACAG

SD-B: CTGTAATAATAGGCATATCTGGTCAATGTCTTCC

are centered around the cryptic splice donor site, and differ from the sequence of GlMD1 at only one base, located in codon 139 of the mdr1 gene, wherein AGG is changed to AGA. This difference inactivates the splice donor site, although the amino acid is not changed. The second set of primers (C185-A and C185-B), which have the following sequences:

C185-A: CTCTAAGATTAAATGAAGGTATTGGTGACAAAATTG

C185-B: CAATTTTGTACCAATACCTTCATTAATCTTAGAG

are centered at the codon 185 mutation of the mdr1 gene and will differ from the sequence of GlMD1 only at the point mutation wherein codon 185 is changed from GTT to GGT. This difference corrects the point mutation in the cDNA sequence (Figure 7) and encodes Gly in the final construction. The third set of primers flank the BstXI and MscI restriction sites. These primers (5'Bst and 3'Msc), which have the following sequences:

5'Bst: ATCGCGGATCCATGGTGGTGGGAACCTTTGGC

3'Msc: CATCCGGAATTCAGCTGACAGTCCAAGAACAGGACTGATG

overlap the BstXI and MscI sites, respectively, and also incorporate another flanking restriction site that will allow the PCR fragment to be conveniently subcloned. Using the technique of "recombinant" PCR, three separate PCR reactions will be initiated, using MD1NA as the template DNA. In these reactions,

a "proofreading" therm stable DNA polymerase, such as Vent polymerase marketed by New England BioLabs, is used to avoid misincorporation of nucleotides, as can occur with high frequency when Taq polymerase is used. In the first PCR reaction, the two primers are 5'Bst and SD-B, and amplify the sequence from the BstXI site to the cryptic splice donor site. In the second PCR reaction, the two primers are SD-A and C185-B, and amplify the sequence from the splice donor to the codon 185 mutation. In the third PCR reaction, the primers are C185-A and 3'Msc, and amplify the sequence from the codon 185 mutation to the MscI site. Following ten cycles of amplification, the product of these three reactions is purified away from unused primers, nucleotides, and buffer. The purified PCR products are mixed together, and then amplified for an additional 10 to 20 cycles with a "proofreading" polymerase such as Vent polymerase, using the 5'Bst and 3'Msc primers. This final PCR reaction results in an amplified PCR product that extends from the BstXI site to the MscI site, and has introduced point mutations into the cryptic splice donor site and the mutant codon 185. Using the flanking restriction sites, the PCR product is digested with BamHI and EcoRI and is subcloned into a pUC based vector prepared by digestion with BamHI and EcoRI using inactivation of the lacZ gene to identify vectors that have incorporated the insert. This construct is referred to as pMD1BM and is used to transform bacteria. Bacteria that contain the plasmid carrying the desired point mutations will be identified by hybridization to allele specific oligonucleotides. MD1BM plasmid DNA isolated from these subclones is then sequenced, using standard M13 sequence primers, to confirm that the desired mutations have been introduced, and that no additional mutations have been created.

After sequencing, a BstXI to MscI fragment is excised from MD1BM and subcloned into a vector prepared by digestion of MD1NA with BstXI and MscI. This plasmid is referred to as MD1NA-C. A NotI to ApaI fragment is excised from MD1NA-C and inserted into a

vector prepared by digestion of G1MD1 with NotI and ApaI. The resulting plasmid is referred to as pG1MD1A, and contains a cDNA for a human mdrl gene that has a conservative point mutation inactivating a cryptic splice donor site, as well as another point mutation that causes a reversion in the amino acid sequence at codon 185 to the wild type sequence.

The cryptic splice acceptor site can be corrected using a similar PCR based strategy. A HindIII to XhoI fragment containing the cryptic splice acceptor can be subcloned directly into the HindIII and XhoI sites of pUC007 (This plasmid is designated pMD1HX). The cryptic splice acceptor is contained within an XmnI to KpnI fragment. Recombinant PCR is then used to introduce a point mutation into the cryptic splice acceptor site. One primer set is required. The first primer (5'Xmn) having the following sequence:

5'Xmn:

ATCGCGGATCCGGAATTATTTCTTTTATTACATTTTTCCTTCAAGGTTTCACATTG

overlaps the XmnI site and extends past the cryptic splice acceptor. This primer will be identical to the sequence in G1MD1 except at one base in codon 733 wherein codon 733 is changed from CAG to CAA, which inactivates the splice acceptor site. The amino acid encoded by codon 733, however, is unchanged. This primer also incorporates a flanking BamHI site to facilitate subcloning of the PCR product. The second primer (3'Kpn), having the following sequence:

3'Kpn:

CTCAAAGAGTTTCTGTATGGTACC

overlaps the KpnI site. These primers are used to amplify a fragment of DNA from pMD1HX with a proofreading polymerase such as Vent polymerase. This PCR product is digested with BamHI and KpnI and subcloned into the BamHI and KpnI sites of a cloning vector such as pUC19 (This plasmid is designated pMD1XK). This plasmid is used to transform bacteria. Subclones containing the "corrected" sequence are identified by allele specific

oligonucleotide hybridization and sequencing as described above. Subsequently, an XmnI to KpnI fragment from pMD1XK is subcloned into the XmnI and KpnI sites of pMD1HX (The resulting plasmid is designated pMD1HX-C). Finally, a HindIII to XhoI fragment from MD1HX-C is subcloned into the HindIII and XhoI sites of pG1MD1 to form pG1MD1B, which contains a cDNA for the mdr1 gene that has a conservative point mutation inactivating a cryptic splice acceptor site.

In order to construct a plasmid that contains a cDNA for an mdr1 gene with mutations introduced into the cryptic splice donor site, codon 185, and the cryptic splice acceptor site, a HindIII to XhoI fragment is excised from pG1MD1B. This fragment is then subcloned into a vector prepared by digesting pG1MD1A with HindIII and XhoI. The resulting plasmid is referred to as pG1MD1AB.

D. Generation of producer cell lines from pG1MD3 and pG1MD1AB.

The vectors pG1MD3 and pG1MD1AB, described in Example 1, which contain the changes in codon 139 (in the splice donor site), in codon 733 (in the splice acceptor site), and in codon 185 (wherein Val 185 is changed to Gly 185), may be placed into a packaging cell line to generate vector particles, and to generate producer cell lines.

Vector producer cell lines are prepared using established protocols. The packaging cell line PE501 (Miller and Rosman, Biotechniques 7:980-990 (1989)) or cell line GP+E86 is plated at a density of 5×10^5 cells per 100 mm plate and the following day purified vector DNA is introduced using standard CaPO_4 precipitation (Wigler et al., Cell 14725-731 (1978)). For each plate of cells to be transfected, 20-40 μg of vector DNA is prepared with a co-precipitate consisting of 0.25M CaCl_2 /1 mM Hepes (pH 7.2) and 140 mM NaCl, 0.75 mM Na_2HPO_4 , 25 mM Hepes (pH 7.2). The DNA/precipitate is allowed to sit at room temperature for 30 min and then added (1 ml/plate) to the cells

in tissue culture medium (DMEM + 10% fetal Bovine serum) for an overnight incubation. The medium is changed to fresh DMEM + serum the following morning. The transfected cells are allowed to grow to near confluence for the next 48 hours, at which point virus supernatant is collected to infect a separate population of PA317 or GP+AM12 vector packaging cell lines at a density of 1×10^5 cells per 100 mm plate seeded 24 hours prior to infection. The standard infection conditions include undiluted virus supernatant, filtered through a 0.2 μ M membrane, to which 8 μ g/ml polybrene is added. After overnight incubation for 16 hours, the medium is changed to DMEM and 10% Fetal Bovine Serum, and grown until such cells are selected with rhodamine 123. The polyclonal population of GP+Aml2 or PA 317 cells are incubated with rhodamine 123 at a concentration of 1 microgram per ml at 37°C for 30 minutes. Following staining, the cells are allowed to efflux the dye for two hours in medium that did not contain rhodamine. The cells are then trypsinized, and 5×10^6 cells are sorted on a Coulter EPICS Elite FACS machine, with excitation at 480nm and fluorescence measured at 525 nm. The bright cells, which did not express p-glycoprotein, are discarded. Cells that are "dull" following rhodamine staining express p-glycoprotein and are collected for further processing.

Example 2

Selection of producer cells generating high titers of viral particles including an mdrl gene.

PA317 (amphotropic) packaging cells were transfected by the CaPO_4 method with pG1MD1. 48 hours later, supernatant from the transfected PA317 cells (Miller, et al., 1989) was filtered and added to GP + E86 (ecotropic) packaging cells in the presence of 6 micrograms per ml of polybrene. 24 hours following infection with this transient supernatant, drug selection with colchicine at a concentration of 60 ng/ml was applied. After two weeks of drug selection, individual drug resistant GP + E86 clones were isolated and characterized for production of recombinant

retrovirus using an RNA slot blot technique (Bodine, et al., Proc. Nat. Acad. Sci., Vol. 87, pgs. 3738-3742 (May 1990)). The ecotropic MDR producer clone with the highest apparent titer, referred to as E4, was used in the subsequent experiments.

Filtered viral supernatant from the ecotropic E4 producer cell line was used to infect the amphotropic packaging cell line GP + Aml2 (Markowitz, et al., Virology, Vol. 167, pgs. 400-406 (1988), Markowitz, et al., J. Virol., Vol. 62, pgs. 1120-1124 (1988)). In this particular experiment, the GP + Aml2 line was exposed to a total of 6 viral supernatants over the course of one week. Following infection, GP + Aml2 cells that had been transduced with the MDR virus and expressed p-glycoprotein were selected by FACS using rhodamine 123 staining to identify transduced cells. The polyclonal population of GP + Aml2 cells was incubated with rhodamine 123 at a concentration of 1 microgram/ml at 37 degrees C for 30 minutes. Following staining, the cells were allowed to efflux the dye for two hours in medium that did not contain rhodamine. The cells were then trypsinized, and 5×10^6 cells were sorted on a Coulter EPICS Elite FACS machine, with excitation at 480nm and fluorescence measured at 525nm. The bright cells, that did not express p-glycoprotein, were discarded. Cells that were "dull" following rhodamine staining express p-glycoprotein and were collected for further processing. In this particular experiment, the "dull" population was further subdivided into three groups ("A", "B", and "C") based on the degree of "dullness." These dull cells were plated at limiting dilution. A total of 1500 wells were plated at two concentrations of one cell per two wells and one cell per five wells. Following two weeks in culture, individual subclones were expanded and characterized for estimated viral titer using the RNA slot blot technique on filtered viral supernatant. Ten clones were identified with the highest production of packaged and secreted viral RNA. These clones were subsequently characterized for viral titer using the rhodamine titrating

protocol. In this protocol, 10 ml of media is conditioned by 5×10^6 producer cells for 24 hours. The supernatant is filtered, and dilutions of the filtered supernatant are used to infect a target cell population, in this case 3T3 cells. To infect 3T3 cells a total of 10 ml of media containing viral supernatant and 6 micrograms per ml of polybrene (or 5 micrograms per ml of protamine sulfate) is added to 10^6 target cells on a 10 cm plate for a period of 48 to 72 hours. The plate of infected 3T3 cells is then stained with rhodamine 123 at 1 microgram/ml at 37 degrees for 30 minutes, and then destained for two hours in media without rhodamine. The plate is then trypsinized and analyzed by FACS to quantitate the proportion of cells that express the "dull" phenotype. Alternatively, the plate of cells can be directly examined by fluorescence microscopy. This information is then used to calculate viral titer. For example, if 0.1 ml of viral supernatant is able to confer the "dull" phenotype to 10 percent of 10^6 cells, then 0.1 ml of viral supernatant contains a minimum of 1×10^5 viral particles, and 1 ml of viral supernatant contains a minimum of 1×10^6 viral particles. As shown in Figure 8A, as the volume of viral supernatant from producer clone G1MD1 A1.2 which contacts the 3T3 cells increases from 0.025 ml to 0.1 ml, and then to 1.0 ml, the height of the left peak, which corresponds to the number of cells having the "dull" phenotype, and which therefore have a lower fluorescence intensity, increases, while there is a decrease in the number of cells having the "bright" phenotype (such cells have a higher fluorescence intensity) as evidenced by the decrease in the size of the right peak. Using this assay, a identified GP + Am clone with a titer of 4×10^6 viral particles per ml was identified. This producer clone was designated G1MD1 A1.2.

Example 3

PA317 cells (Miller, et al., 1989), obtained from the ATCC and selected in HMT medium (following the protocol suggested by the ATCC) were transduced with viral supernatant from the

ecotropic E4 producer cell line obtained as hereinabove described in Example 2. The PA317 packaging cell line was serially infected with E4 viral supernatant over the course of 10 days until 100% of the cells in the population expressed the rhodamine "dull" phenotype. The phenotype was evaluated by fluorescence microscopy and FACS analysis on aliquots of the population. A total of 13 exposures to viral supernatants were used. The population of infected PA317 cells were directly plated at limiting dilution. A total of 1,500 wells were plated at dilutions of 1:3 and 1:5 cells per well. Individual clones were isolated and subsequently scored for viral RNA production using the slot blot assay. The subclones with the highest production of viral RNA were then analyzed by the rhodamine titrating assay hereinabove described in Example 2. As shown in Figure 8B, as the volume of viral supernatant from producer clone PA15 which contacts the 3T3 cells increases from 0.025 ml to 0.1 ml and then to 1.0 ml, the height of the left peak, which corresponds to the number of cells having the "dull" phenotype, and which therefore have a lower fluorescence intensity, increases, while there is a decrease in the number of cells having the "bright" phenotype (such cells have a higher fluorescence intensity) as evidenced by the decrease in the size of the right peak. One subclone, PA15, was identified which had a titer of 5×10^6 viral particles per ml.

Example 4

Bone marrow cells were harvested from the posterior iliac crests and femurs of 3-4 kg juvenile rhesus monkeys (Proc. Natl. Acad. Sci. USA, Vol. 87, pgs. 3738-3742 (1990)). The cells are aspirated into Dulbecco's Modified Eagle's Medium (DMEM) containing 2% fetal calf serum and 10 units of heparin/ml. Five days before the bone marrow harvest, the animals receive 5-fluorouracil (70mg/kg) as a single intravenous bolus. During the bone marrow harvest procedure, the animal receives 100ml of autologous red cells to replace the volume of blood that is

removed. Prior to the bone marrow harvest, an in-dwelling central venous catheter is established by standard surgical techniques.

After harvest, the bone marrow was diluted with Hank's Buffered Saline Solution (HBSS) and passed through wire mesh to remove clumps of cells. Mononuclear cells were isolated by density gradient centrifugation using Ficoll-Paque. Cells expressing the CD34 antigen are isolated by immunoselection (Science, Vol. 242, pgs. 919-922 (1988)) using the magnetic bead technology. $2-4 \times 10^9$ total bone marrow cells are recovered by the harvest procedure. Approximately 20% of these cells are recovered as the mononuclear cell preparation and 2-3% of these cells are recovered by immunoselection. Thus a total of $2-4 \times 10^7$ CD34 cells are obtained at the time of bone marrow harvest. These were incubated at an initial concentration of 5×10^5 cells/ml for 36 hours in DMEM, 15% fetal calf serum, penicillin-streptomycin, and 2X glutamine. The following hematopoietic growth factors were added: Stem Cell Factor (100ng/ml), interleukin-3 (50ng/ml) and interleukin-6 (50ng/ml). After 36 hours, the cells were recovered by centrifugation and resuspended at 5×10^5 cells/ml in fresh medium conditioned by the retroviral producer clone G1MD1 A1.2 prepared as described in Example 2. This medium includes hematopoietic growth factors as specified above plus protamine sulfate at a concentration of 5ug/ml. Every twelve hours the cells were recovered by centrifugation and resuspended in fresh virus conditioned medium with growth factors and protamine. After 72 hours of incubation in the presence of retroviral vector particles, the cells were recovered by centrifugation, resuspended in DMEM or HBSS containing 2% fetal calf serum and 10 units of heparin per ml, and reinfused into the transplant recipient. During the in vitro culture, the cells expand approximately 10 fold so that $2-4 \times 10^6$ cells are returned to the animal.

Initially, cells were analyzed after in vitro incubation for evidence of gene transfer and expression. DNA was recovered and subjected to analysis by the polymerase chain reaction methodology using primers (MDR Primer 7, which has the sequence 5'-GCCCACATCATCATGATC-3' and MDR Primer 8, which has the sequence 5'-GTCTCCTACTTTAGTGCT-3') specific for the human mdr1 coding sequences. Based on comparison to signal intensity obtained with a retroviral producer clone containing a single copy of the retroviral genome, we concluded that 10% of the cells had undergone transduction. The Rhodamine efflux assay hereinabove described was performed confirming that, when compared to a mock infected control sample, 10% of the CD34 selected, transduced cells expressed the mdr1 gene product.

The transplant recipients received daily intravenous fluids, broad spectrum antibiotics, antifungal agents, hyperalimentation solution and blood products as needed to maintain an optimal physiological condition. Daily blood counts and blood chemistries were obtained to monitor recovery. On day 1 following transplantation, granulocyte colony-stimulating factor was given at a dose of 5ug/kg/day by continuous intravenous infusion. When recovery to a leukocyte count of 1,000-3,000/mm³ occurs (day 14-22), blood samples are obtained for detection of the mdr1 gene and its expression.

DNA was purified from the total population of blood leukocytes after lysis of the red cells, and isolation of nuclei from leukocytes. This DNA was analyzed by the polymerase chain reaction methodology using the P7 and P8 primers described above. In the first animal transplanted, approximately 1% of the cells contained the retroviral genome whereas in the second animal 8% of the cells contained the transferred sequences.

It is to be understood, however, that the scope of the present invention is not to be limited to the specific embodiments described above. The invention may be practiced

other than as particularly described and still be within the scope of the accompanying claims.

PATAP859

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: McDonagh, Kevin T.
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Tolstoshev, Paul

(ii) TITLE OF INVENTION: IMPROVED EXPRESSION OF HUMAN
MULTIDRUG RESISTANCE GENES AND IMPROVED
SELECTION OF CELLS TRANSDUCE WITH SUCH GENES

(iii) NUMBER OF SEQUENCES: 18

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(v) COMPUTER READABLE FORM:

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-30-

- (C) OPERATING SYSTEM: PC-DOS
- (D) SOFTWARE: DW4.V2

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- (A) APPLICATION NUMBER:
- (B) FILING DATE:

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 9 bases
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: singular
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE:

- (A) DESCRIPTION: Genomic DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

CAGGTATGC

9

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 16 bases
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: singular
- (D) TOPOLOGY: linear

-31-

(ii) MOLECULE TYPE:

(A) DESCRIPTION: G nomic DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

ACATTTTTC TTCAGG

16

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 50 bases
(B) TYPE: nucleic acid
(C) STRANDEDNESS: singular
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE:

(A) DESCRIPTION: Genomic DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

ACTGAACTCT GGTAACTCC ACTCGAGCAC AGATCTGGAC ATCGATACTC

50

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 30 bases
(B) TYPE: nucleic acid
(C) STRANDEDNESS: singular
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE:

(A) DESCRIPTION: Genomic DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

TCTGTACTGG TCTATACGGA TAATAATGTC

30

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 30 bases
(B) TYPE: nucleic acid
(C) STRANDEDNESS: singular
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE:

(A) DESCRIPTION: Genomic DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5

CTAATTACTT CCATAACCAC TGTTTTAACC

30

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 30 bases
(B) TYPE: nucleic acid
(C) STRANDEDNESS: singular
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE:

(A) DESCRIPTION: Genomic DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

GTAAAAAGGA AGTTCCAAAG TGTAACCGT

30

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 33 bases
(B) TYPE: nucleic acid
(C) STRANDEDNESS: singular
(D) TOPOLOGY: linear

-33-

(ii) MOLECULE TYPE:

(A) DESCRIPTION: DNA primer

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

GGAAGACATG ACCAGATATG CCTATTATTA CAG

33

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 33 bases
(B) TYPE: nucleic acid
(C) STRANDEDNESS: singular
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE:

(A) DESCRIPTION: DNA primer

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

CTGTAATAAT AGGCATATCT GGTCATGTCT TCC

33

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 35 bases
(B) TYPE: nucleic acid
(C) STRANDEDNESS: singular
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE:

(A) DESCRIPTION: DNA primer

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

CTCTAAGATT AATGAAGGTA TTGGTGACAA AATTG

35

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 35 bases
(B) TYPE: nucleic acid
(C) STRANDEDNESS: singular
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE:

(A) DESCRIPTION: DNA primer

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

CAATTTTGTG ACCAATACCT TCATTAATCT TAGAG

35

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 31 bases
(B) TYPE: nucleic acid
(C) STRANDEDNESS: singular
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE:

(A) DESCRIPTION: DNA primer

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

ATCGCGGATC CATGGTGGTG GGAAC TTTGG C

31

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 40 bases
(B) TYPE: nucleic acid
(C) STRANDEDNESS: singular
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE:

(A) DESCRIPTION: DNA primer

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

CATCCGGAAT TCAGCTGACA GTCCAAGAAC AGGACTGATC

40

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 58 bases
(B) TYPE: nucleic acid
(C) STRANDEDNESS: singular
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE:

(A) DESCRIPTION: DNA primer

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

ATCGCGGATC CGGAATTATT TCTTTTATTA CATTTTTCCT TCAAGGTTTC ACATTG 58

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 24 bases
(B) TYPE: nucleic acid
(C) STRANDEDNESS: singular
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE:

(A) DESCRIPTION: DNA primer

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

CTCAAAGAGT TTCTGTATGG TACC

24

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 18 bases
(B) TYPE: nucleic acid
(C) STRANDEDNESS: singular
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE:

- (A) DESCRIPTION: DNA primer

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

GCCCACATCA TCATGATC

18

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 18 bases
(B) TYPE: nucleic acid
(C) STRANDEDNESS: singular
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE:

- (A) DESCRIPTION: DNA primer

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

GTCTCCTACT TTAGTGCT

18

(2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 53 base pairs, having 4 unpaired bases at the 5' end, and 2 unpaired bases at 3' end.

(B) TYPE: nucleic acid
(C) STRANDEDNESS: singular
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE:

(A) DESCRIPTION: Plasmid DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

AATTCGCGGC CGCTACGTAG TCGACGGATC CCTCGAGAAG CTTGGGCCCA T
CGGCGG GCGATGCATC AGCTGCCTAG GGAGCTCTTC GAACCCGGGT AGC 53

(2) INFORMATION FOR SEQ ID NO:18:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 4669 bases
(B) TYPE: nucleic acid
(C) STRANDEDNESS: singular
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE:

(A) DESCRIPTION: Genomic DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

CATACTCTAT TCAGATATTC TCCAGATTCC TAAAGATTAG AGATCATTTT TCATTCTCCT 60
AGGAGTACTC ACTTCAGGAA GCAACCAGAT AAAAGAGAGG TGCAACGGAA GCCAGAACAT 120
TCCTCCTGGA AATTC AACCT GTTTCGCAGT TTCTCGAGGA ATCAGCATTC AGTCAATCCG 180
GGCCGGGACC AGTCATCTGT GGTGAGGCTG ATTGGCTGGG CAGGAACAGC GCCGGGGCGT 240
GGGCTGAGCA CACCGCTTCG CTCTCTTTGC CACAGGAAGC CTGAGCTCAT TCGAGTAGCG 300
GCTCTTCCAA GCTCAAAGAA GCAGATCCG CTGTTTCGTT CTTTAGGTC TTTCCACTAA 360
AGTCGGAGTA TCTTCTTCCA AGATTTCACG TCTTGGTGGC CGTTCCAAGG AGCGCGAGGT 420

CGGGATGGAT CTTGAAGGGG ACCGCAATGG AGGAGCAAAG AAGAAGAACT TTTTAAACT	480
GAACAATAAA AGTGAAAAAG ATAAGAAGGA AAAGAAACCA ACTGTCAGTG TATTTTCAAT	540
GTTTCGCTAT TCAAATTGGC TTGACAAGTT GTATATGGTG GTGGGAACTT TGGCTGCCAT	600
CATCCATGGG GCTGGACTTC CTCTCATGAT GCTGGTGTTT GGAGAAATGA CAGATATCTT	660
TGCAAATGCA GGAAATTTAG AAGATCTGAT GTCAAACATC ACTAATAGAA GTGATATCAA	720
TGATACAGGG TTCTTCATGA ATCTGGAGGA AGACATGACC AGGTATCCCT ATTATTACAG	780
TGGAATTGGT GCTGGGGTGC TGGTTGCTGC TTACATTGAG GTTTCATTTT GGTGCCTGGC	840
AGCTGGAAGA CAAATACACA AAATTAGAAA ACAGTTTTTT CATGCTATAA TGCGACAGGA	900
GATAGGCTGG TTTGATGTGC ACGATGTTGG GCAGCTTAAC ACCCGACTTA CAGATGATGT	960
CTCTAAGATT AATGAACTTA TTGGTGACAA AATTGGAATG TTCTTTCAGT CAATGGCAAC	1020
ATTTTTCACT GGGTTTATAG TAGGATTTAC ACGTGTTGG AAGCTAACCC TTGTGATTTT	1080
GGCCATCAGT CCTGTTCTTG GACTGTCAGC TGCTGTCTGG GCAAAGATAC TATCTTCATT	1140
TACTGATAAA GAACTCTTAG CGTATGCAAA AGCTGGAGCA GTAGCTGAAG AGGTCTTGGC	1200
AGCAATTAGA ACTGTGATTG CATTTGGAGG ACAAAGAAA GAACTTGAAA GGTACAACAA	1260
AAATTTAGAA GAAGCTAAAA GAATTGGGAT AAAGAAAGCT ATTACAGCCA ATATTTCTAT	1320
AGGIGCTGCT TTCCTGCTGA TCTATGCATC TTATGCTCTG GCCTTCTGGT ATGGGACCAC	1380
CTTGGTCCTC TCAGGGGAAT ATTCTATTGG ACAAGTACTC ACTGTATTCT TTTCTGTATT	1440
AATTGGGGCT TTTAGTGTTG GACAGGCATC TCCAAGCATT GAAGCATTG CAAATGCAAG	1500
AGGAGCAGCT TATGAAATCT TCAAGATAAT TGATAATAAG CCAAGTATTG ACAGCTATTC	1560
GAAGAGTGGG CACAAACCAG ATAATATTAA GGGAAATTTG GAATTCAGAA ATGTTCACTT	1620
CAGTIACCCA TCTCGAAAAG AAGTTAAGAT CTTGAAGGGC CTGAACCTGA AGGTGCAGAG	1680
TGGGCAGACG GTCCCCCTGG TTGGAAACAG TGGCTGTGGG AAGAGCACAA CAGTCCAGCT	1740
GATGCAGAGG CTCTATGACC CCACAGAGGG GATGGTCAGT GTTCATGGAC AGGATATTAG	1800
GACCATAAAT GTAAGGTTTC TACGGGAAAT CATTGGTGTG GTGAGTCAGG AACCTGTATT	1860
GTTTGCCACC ACGATAGCTG AAAACATTG CTATGGCCGT GAAAATGTCA CCATGGATGA	1920
GATTGAGAAA GCTGTCAAGG AAGCCAATGC CTATGACTTT ATCATGAAAC TGCCTCATAA	1980

ATTGACACC CTGGTTGGAG AGAGAGGGCC CCAGTTGAGT GGTGGGCAGA AGCAGAGGAT	2040
CGCCATTGCA CGTGCCCTGG TTCGCAAGCC CAAGATCCTC CTGCTGGATG AGGCCACGTC	2100
AGCCTTGGAC ACAGAAAGCG AAGCAGTGGT TCAGGTGGCT CTGGATAAGG CCAGAAAAGG	2160
TCGGACCACC ATTGTGATAG CTCATCGTTT GTCTACAGTT CGTAATGCTG ACGTCATCGC	2220
TGGTTTCGAT GATGGAGTCA TTGTGGAGAA AGGAAATCAT GATGAACTCA TGAAAGAGAA	2280
AGGCTTTTAC TTCAAACCTG TCACAACTCA GACAGCAGGA AATGAAGTTG AATTAGAAAA	2340
TGCAGCTGAT GAATCCAAAA GTGAAATTGA TGCCTTGGAA ATGTCTTCAA ATGATTCAAG	2400
ATCACGTCTA ATAAGAAAAA GATCAACTCG TAGGAGTGTC CGTGGATCAC AACCCCAAGA	2460
CAGAAAGCTT AGTACCAAAG AGGCTCTGGA TGAAAGTATA CCTCCAGTTT CCTTTTGGAG	2520
GATTATGAAG CTAAATTTAA CTGAATGGCC TTATTTTGTT GTGGTGTAT TTTGTGCCAT	2580
TATAAATGGA GGCCTGCAAC CAGCATTGTC AATAATATTT TCAAAGATTA TAGGGGTTTT	2640
TACAAGAATT GATGATCCTG AAACAAAACG ACAGAATACT AACTTGTTTT CACTATTGTT	2700
TCTAGCCCTT GGAATTATTT CTTTATTAC ATTTTTCCTT CAGGGTTTCA CATTGGCAA	2760
AGCTGGAGAG ATCCTCACCA AGCGGCTCCG ATACATGGTT TTCCGATCCA TGCTCAGACA	2820
GGATGTGAGT TGGTTTGATG ACCCTAAAAA CACCACTGGA GCATTGACTA CCAGGCTCGC	2880
CAATGATGCT GCTCAAGTTA AAGGGGCTAT AGGTTCCAGG CTTGCTGTAA TTACCCAGAA	2940
TATAGCAATT CTTGGGACAG GAATAATTAT ATCCTTCATC TATGGTTGGC AACTAACACT	3000
GTTACTCTTA GCAATTGTAC CCATCATTGC AATAGCAGGA GTTGTGAAA TGAAAATGTT	3060
GTCTGGACAA GCACTGAAAG ATAAGAAAGA ACTAGAAGGT GCTGGGAAGA TCGCTACTGA	3120
AGCAATAGAA AACTTCCGAA CCGTTGTTTC TTTGACTCAG GAGCAGAAGT TTGAACATAT	3180
GTATGCTCAG AGTTTGCAGG TACCATACAG AAACTCTTTG AGGAAAGCAC ACATCTTTGG	3240
AATTACATTT TCCTTCACCC AGGCAATGAT GTATTTTTC TATGCTGGAT GTTCCGGTT	3300
TGGAGCCTAC TTGGTGGCAC ATAAACTAAT GAGCTTTGAG GATGTTCTGT TAGTATTTTC	3360
AGCTGTTGTC TTTGGTGCCA TGGCCGTGGG GCAAGTGAGT TCATTTGCTC CTGACTATGC	3420
CAAAGCCAAA ATATCAGCAG CCCACATCAT CATGATCATT GAAAAAAGCC CTTTGATTGA	3480
CAGCTACAGC ACGGAAGGCC TAATGCCGAA CACATTGGAA GGAAATGTCA CATTGGTGA	3540

AGTTGTATTC AACTATCCCA CCCGACCGGA CATCCCAGTC CTTCAGGGAC TGAGCCTGGA	3600
GGTGAAGAAG GGCCAGACGC TGGCTCTGGT GGGCAGCAGT GGCTGTGGGA AGAGCACAGT	3660
GGTCCAGCTC CTGGAGCGGT TCTACGACCC CTTGGCAGGG AAAGTGCTGC TTGATGGCAA	3720
AGAAATAAAG CGACTGAATG TTCAGTGGCT CCGAGCACAC CTGGGCATCG TGTCCCAGGA	3780
GCCCATCCTG TTTGACTGCA GCATTGCTGA GAACATTGCC TATGGACACA ACAGCCGGGT	3840
GGTGTACAG GAAGAGATCG TGAGGGCAGC AAAGGAGGCC AACATACATG CCTTCATCGA	3900
GTCCTGCCT AATAAATATA GCACTAAAGT AGGAGACAAA GGAATCAGC TCTCTGGTGG	3960
CCAGAAACAA CGCATTGCCA TAGCTCGTGC CTTGTTAGA CAGCCTCATA TTTTGCTTTT	4020
GGATGAAGCC ACGTCAGCTC TGGATACAGA AAGTGAAAAG GTTGTCCAAG AAGCCCTGGA	4080
CAAACCCAGA GAAGGCCGCA CCTGCATTGT GATTGCTCAC CGCCTGTCCA CCATCCAGAA	4140
TGCAGACTTA ATAGTGGTGT TTCAGAATGG CAGAGTCAAG CAGCATGGCA CGCATCAGCA	4200
GCTGCTGGCA CAGAAAGGCA TCTATTTTTT AATGGTCAGT GTCCAGCCTG GAACAAAGCG	4260
CCAGTGAAT CTGACTGTAT GAGATGTTAA ATACTTTTTA ATATTTGTTT AGATATGACA	4320
TTTATTCAAA GTTAAAAGCA AACACTTACA GAATTATGAA GAGGTATCTG TTTAACATTT	4380
CCTCACTCAA CTTCAGAGTC TTCAGAGACT TCGTAATTAA AGGAACAGAG TGAGAGACAT	4440
CATCAAGTGG AGAGAAATCA TAGTTTAAAC TGCATTATAA ATTTTATAAC AGAATTAAAG	4500
TAGATTTTAA AAGATAAAAT GTGTAATTTT GTTTATATTT TCCCATTGG ACTGTAACTG	4560
ACTGCCTTGC TAAAAGATTA TAGAAGTAGC AAAAAGTATT GAAATGTTTG CATAAAGTGT	4620
CTATAATAAA ACTAACTTT CATGTGAAAA AAAAAAAAAA AAAAAAAAAA	4669

WHAT IS CLAIMED IS:

1. Human mdr1 DNA or RNA which encodes p-glycoprotein or an analogue or fragment of such DNA or RNA which encodes an iso-form of p-glycoprotein which confers multidrug resistance, said DNA or RNA including at least one splice site, wherein at least one base in the splice site has been changed to a different base which thereby inactivates the splice site.
2. The DNA or RNA of Claim 1 wherein said DNA or RNA is a DNA or RNA sequence for a human mdr1 gene which encodes p-glycoprotein wherein at least one base in a splice site of said DNA encoding p-glycoprotein is changed.
3. The DNA sequence of Claim 2 wherein said at least one base is in a splice donor site.
4. The DNA sequence of Claim 2 wherein said at least one base is in a splice acceptor site.
5. The DNA sequence of Claim 2 wherein at least one base in a splice donor site is changed and at least one base in a splice acceptor site is changed.
6. The DNA sequence of Claim 2 wherein said at least one base is in a splice donor site, and is at least one of guanine or thymine contained in said splice donor site, and wherein said guanine and thymine are adjacent.
7. The DNA sequence of Claim 2 wherein said at least one base is in a splice acceptor site, and is at least one of adenine or guanine contained in said splice acceptor site, and wherein said adenine and guanine are adjacent.
8. The DNA sequence of Claim 2 wherein said at least one base in a splice site is changed such that a codon encoding an amino acid is changed to a different codon encoding the same amino acid.
9. The DNA sequence of Claim 8 wherein codon 139 is changed from AGG to AGA.
10. The DNA sequence of Claim 8 wherein codon 733 is changed from CAG to CAA.

11. The DNA sequence of Claim 8 wherein codon 139 is changed from AGG to AGA and codon 733 is changed from CAG to CAA.

12. DNA encoding a protein which provides for multidrug resistance wherein said DNA includes the following sequence:

CAGGTATGC

wherein at least one base is changed to a different base.

13. The DNA of Claim 12 wherein at least one base of the GT doublet is changed.

14. DNA encoding a protein which provides for multidrug resistance wherein said DNA includes the following sequence:

ACATTTTTCCTTCAGG

wherein at least one base is changed to a different base.

15. The DNA of Claim 14 wherein at least one base of the AG doublet is changed.

16. DNA encoding a protein which provides for multidrug resistance wherein said DNA includes a first sequence:

CAGGTATGC, and a second sequence:

ACATTTTTCCTTCAGG, wherein at least one base of said first sequence is changed, and at least one base of said second sequence is changed.

17. The DNA of Claim 16 wherein at least one base of the GT doublet of the first sequence is changed and at least one base of the AG doublet of the second sequence is changed.

18. The DNA sequence of Claim 2 wherein said DNA sequence is further mutated such that a portion of the 5' untranslated region of the DNA has been removed.

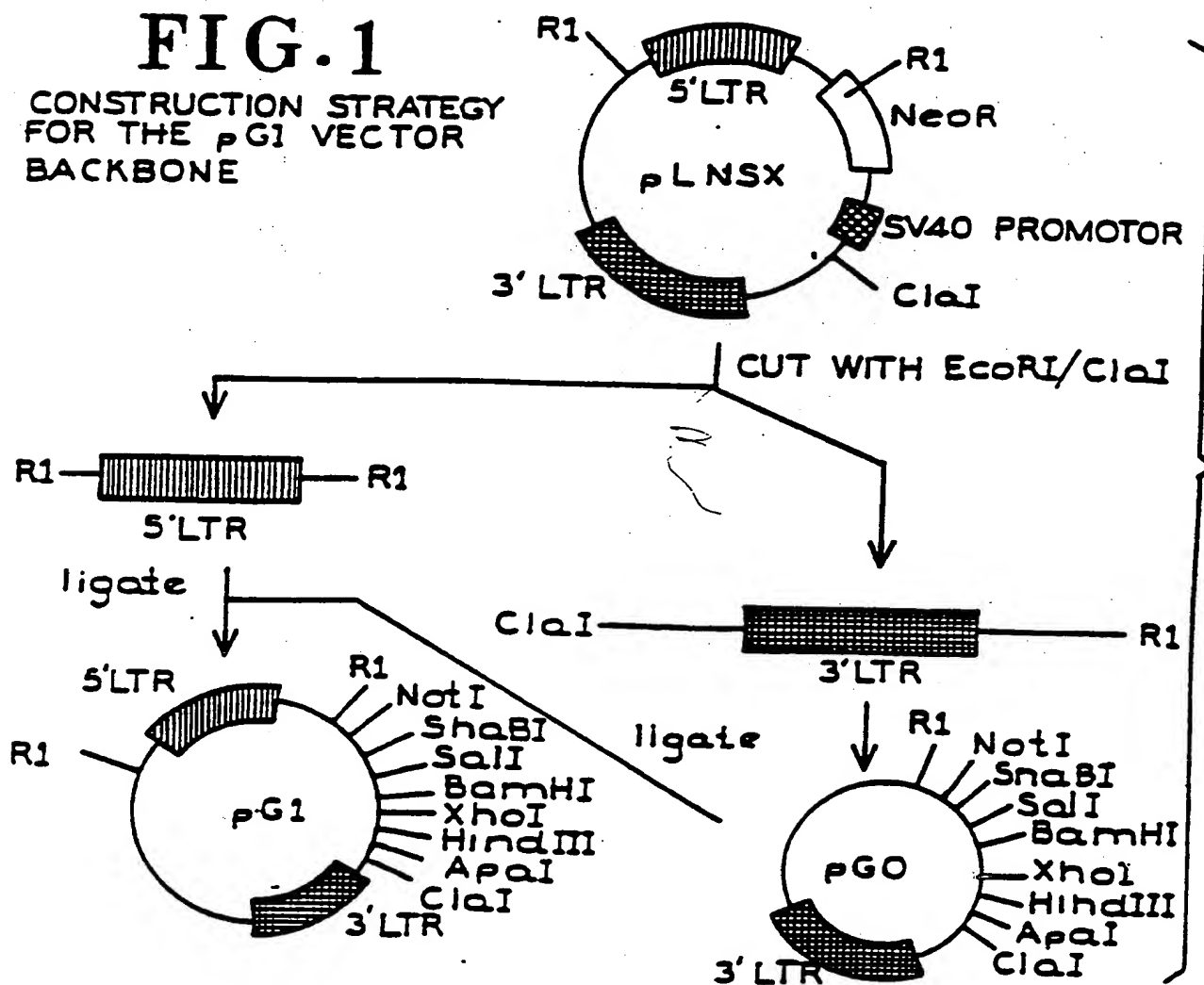
19. The DNA sequence of Claim 2 wherein said DNA sequence is further mutated such that a portion of the 3' untranslated region of the DNA has been removed.

20. The DNA sequence of Claim 2 wherein said DNA sequence is further mutated such that a portion of the 5' untranslated region of the DNA and a portion of the 3' untranslated region of the DNA have been removed.

21. An expression vehicle including the DNA or RNA of Claim 1.

22. The expression vehicle of Claim 21 wherein the expression vehicle is a retroviral vector.
23. Cells genetically engineered with the retroviral vector of Claim 22.
24. A method of identifying cells which overexpress the human mdr1 gene in a cell population that has been transduced with an expression vehicle including a human mdr1 gene, comprising:
 contacting said cell population with a staining material;
 and
 identifying cells which overexpress the human mdr1 gene based on differentiation in color among the cells of said cell population.
25. The method of Claim 24 wherein said staining material is a fluorescent dye.
26. The method of Claim 25 wherein said fluorescent dye is rhodamine 123.
27. Primate cells genetically engineered with DNA or RNA encoding a protein which provides multidrug resistance.
28. The cells of Claim 27 wherein said primate cells are human cells.
29. The cells of Claim 28 wherein said human cells are bone marrow cells.
30. The cells of Claim 28 wherein said human cells are hematopoietic progenitor cells.
31. The cells of Claim 28 wherein the cells are repopulating hematopoietic stem cells.

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FIG. 1**CONSTRUCTION STRATEGY
FOR THE pG1 VECTOR
BACKBONE**

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FIG. 2

SEQUENCE OF THE MULTIPLE CLONING SITE IN THE pGI PLASMID

<u>1/2 EcoRI</u>	<u>NotI</u>	<u>SnaBI</u>	<u>SacI</u>	<u>BamHI</u>	<u>XhoI</u>	<u>HindIII</u>	<u>ApaI</u>
AATTC	GCGGCCGC	TACGTA	GTCGAC	GGATCC	CTCGAG	AAGCTT	GGGCCC
G	CGCCGGCG	ATGCAT	CAGCTG	CCTAGG	GAGCTC	TTCGAA	CCCGGG

1/2 ClaI

AT

TAGC

5'

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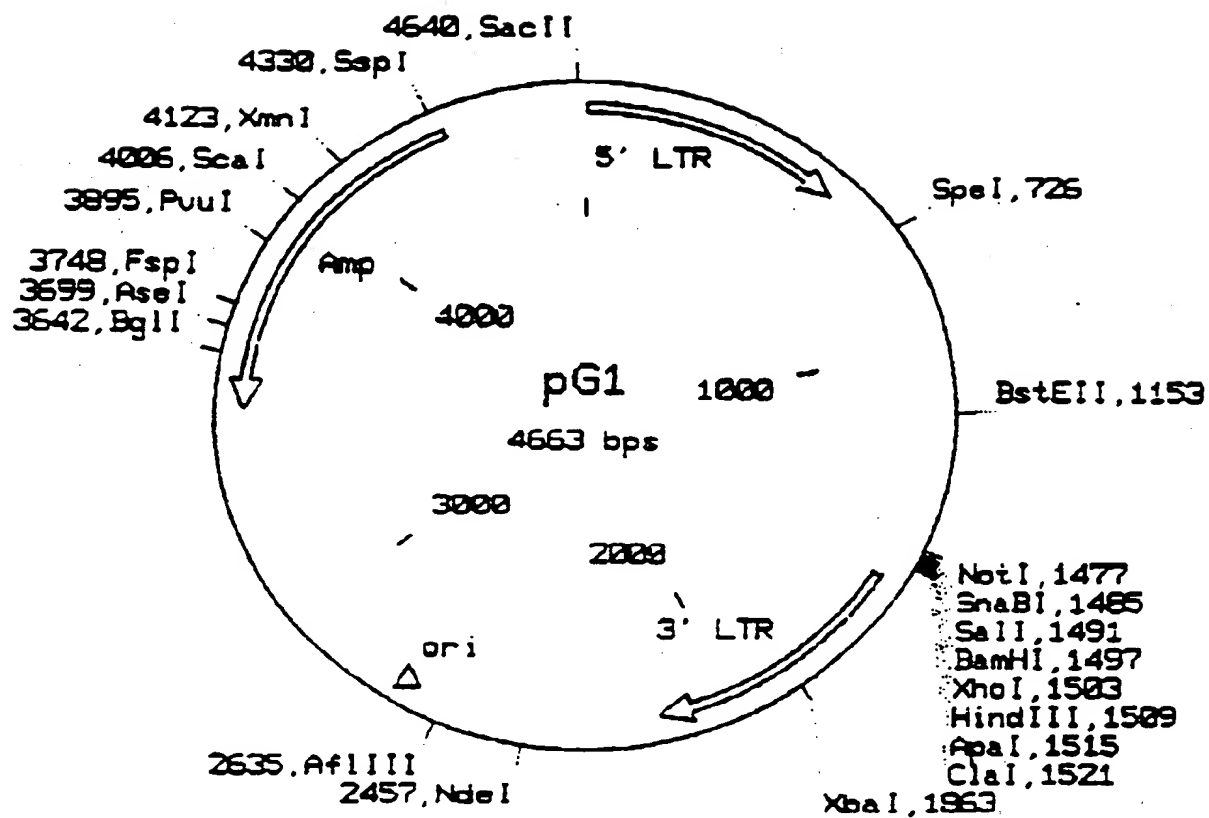


Fig. 3

cdNA Sequence of Human mdrl Gene

1	CTTACTCTAT	TTCAGATATTC	TCCAGATTC	TAAAGATTAG	AGATCATTTTC
51	TCATTCTCCT	AGGAGTACTC	ACTTCAGUA	GCNAACCAGAT	AAAAGAGAGCG
101	TGCAACGGAA	GCCAGAACAT	TCCCTCTGUA	AAATCAACCT	GTTCGCGAGT
151	TTCTCGAGGA	ATCAGCATTC	ACTCAATCCG	GGCCGGGAGC	AGTCATCTGT
201	GGTGAGGCTG	ATTGGCTCGG	CAAGAACAGC	GGCGGGCGT	GGGCTGAGCA
251	CAGCGCTTCG	CTCTCTTTGC	CACAGCMAGC	CTTACCTCAT	TCCAGTAGCG
301	GGCTCTTCCA	GCTCAAGAA	GCAGAGGCCG	CTCTTCGTT	CCTTAGGTC
351	TTTCCACTAA	AGTCGGAGTA	TCTTCTTCCA	AGATTTCAGC	TCTTGGTGGC
401	CGTCCCAAGG	AGGCGAGGT	CGGG		
425	ATG GAT CTT GAA GGG GAC CGC AAT GUA GGA GGA GGA AAG AAG AAG				
	Met Asp Leu Glu Gly Asp Arg Asn Gly Gly Ala Lys Lys Lys Lys				
467	AAC TTT TTT AAA CTG AAC AAT AAA AGT GAA AAA CAT AAG AAG				
	Asn Phe Phe Lys Leu Asn Asn Lys Ser Glu Lys Asp Lys Lys				
509	GAA AAG AAG CCA ACT CTC AGT GTA TTT TCA ATG TTT CGC TAT				
	Glu Lys Lys Phe Thr Val Ser Met Phe Arg Tyr				
551	TCA AAT TCG CTT GAA AAG TTC TAT ATG CTC GTC GGA ACT TTG				
	Ser Asn Trp Leu Asp Lys Leu Tyr Met Val Val Gly Thr Leu				
593	GCT GGC ATC ATC CAT GAG GGT GCA CTT CCT CTC ATG ATG CTC				
	Ala Ala Ile Ile His Gly Ala Gly Leu Pro Leu Met Met Leu				
615	GTG TTT GGA GAA ATG ACA GAT ATC TTT GCA AAT GCA GGA AAT				
	Val Phe Gly Glu Met Thr Asp Ile Phe Ala Asn Ala Gly Asn				

Fig. 4

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677 TTA GAA GAT CTC ATG TCA AAC ATC ACT AAT ACA AGT GAT ATC 718
 Leu Gln Asp Leu HET Ser Asn Ile Thr Asn Arg Ser Asp Ile
 719 AAT GAT ACA GGG TTC ATG AAT CTG GAG GAA CAC ATG ACC 760
 Asn Asp Thr Gly Phe Phe HET Asn Leu Gln Gln Asp HET Thr
 761 AGC TAT GGC TAT TAT TAC AAT GCA ATT GCT GGT GGG GTC CTC 802
 Arg Tyr Ala Tyr Tyr Ser Gly Ile Gly Ala Gly Val Leu
 803 GCT GCT GCT TAC ATT CAG GGT TCA TTT TGG TCG CTC GCA GCT 844
 Val Ala Ala Tyr Ile Gln Val Ser Phe Thr Cys Leu Ala Ala
 845 GCA AUA CAA ATA CAC AAA ATT ACA AAA CAG TTT TTT CAT GCT 886
 Gly Arg Gln Ile His Lys Ile Arg Lys Gln Phe Phe His Ala
 887 ATA ATG CGA CAG GAG ATA GGC TGG TTT CAT CTC CAC CAT GTT 928
 Ile HET Arg Gln Gln Ile Gly Thr Phe Asp Val His Asp Val
 929 GGC CAG CTT AAC ACC GCA CTT ACA GAT CAT CTC TCT AAG ATT 970
 Gly Gln Lys Asn Thr Arg Leu Thr Asp Asp Val Ser Lys Ile
 971 AAT GAA GGT TTT GGT GAC AAA ATT GGA ATG TTC TTT CAG TCA 1012
 Asn Gln Val Phe Gly Asp Lys Ile Gly HET Phe Phe Gln Ser
 1013 ATG GCA ACA TTT TTC ACT GGC TTT ATA CTA GCA TTT ACA GGT 1054
 HET Ala Thr Phe Phe Thr Gly Phe Ile Val Gly Phe Thr Arg
 1055 GGT TGG AAG CTA ACC CTT CTC ATT TTG GCC ATC AGT CCT GTT 1096
 Gly Thr Lys Leu Thr Leu Val Ile Leu Ala Ile Ser Pro Val

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1097 CTT GGA CTG TCA GGT GGT GTC TCG GCA AAG ATA CTA TCT TCA 1118
 Leu Gly Leu Ser Ala Ala Val Val Trp Ala Lys Ile Leu Ser Ser
 1119 TTT ACT GAT AAA GAA CTC TTA GCG TAT GCA AAA GCT GCA GCA 1100
 Phe Thr Asp Lys Glu Leu Leu Ala Tyr Ala Lys Ala Gly Ala
 1181 GTA GCT GAA GAG GTC TTC GCA GCA ATT AGA ACT GTC ATT GCA 1222
 Val Ala Glu Glu Val Leu Ala Ala Ile Arg Thr Val Ile Ala
 1221 TTT GGA GGA CAA AAG AAA GAA CTT GAA AGC TAC AAC AAA AAT 1264
 Phe Gly Gly Glu Lys Lys Glu Leu Glu Arg Tyr Asn Lys Asn
 1265 TTA GAA GAA GCT AAA ACA ATT GCG ATA AAG AAA GCT ATT ACA 1306
 Leu Glu Glu Ala Lys Arg Ile Gly Ile Lys Lys Ala Ile Thr
 1307 GCC AAT ATT TCT ATA GGT GCT GCT TTC CTG ATC TAT TAT GCA 1348
 Ala Asn Ile Ser Ile Gly Ala Ala Phe Leu Leu Ile Tyr Ala
 1319 TCT TAT GCT CTG GCC TTC TCG TAT GCG ACC ACC TTG GTC CTC 1390
 Ser Tyr Ala Leu Ala Phe Trp Tyr Gly Thr Thr Leu Val Leu
 1391 TCA GCG GAA TAT TCT ATT GGA CAA GTA CTC ACT GTA TTC TTT 1432
 Ser Gly Glu Tyr Ser Ile Gly Glu Val Leu Thr Val Phe Phe
 1433 TCT GTA TTA ATT GCG GCT TTT AGT GTT GCA CAG GCA TCT CCA 1474
 Ser Val Leu Ile Gly Ala Phe Ser Val Gly Glu Ala Ser Pro
 1475 AGC ATT GAA GCA TTT GCA AAT GCA AGA GCA GCT TAT GAA 1516
 Ser Ile Glu Ala Phe Ala Asn Ala Arg Gly Ala Ala Tyr Glu

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1517 ATC TTC AAC ATA ATT GAT AAT AAG CCA AGT ATT GAC AGC TAT 1530
 Ile Phe Lys Ile Ile Asp Asn Lys Pro Ser Ile Asp Ser Tyr
 1559 TCG AAG AGT GCG CAC AAA CCA GAT AAT ATT AAG GGA AAT TTG 1600
 Ser Lys Ser Gly His Lys Lys Pro Asp Asn Ile Lys Gly Asn Leu
 1601 GAA TTC AGA AAT GTT CAC TTC AGT TAC CCA TCT CGA AAA GAA 1642
 Glu Phe Arg Asn Val His Phe Ser Tyr Pro Ser Arg Lys Glu
 1641 GTT AAG ATC TTC AAG GGC CTC AAC CTC AAG GTC CAG CTT GCG 1684
 Val Lys Ile Leu Lys Gly Leu Asn Leu Lys Val Glu Ser Gly
 1685 CAG ATC CTC GGC CTT GTT GGA AAC AGT GGC TGT GCG AAG AGC 1726
 Glu Thr Val Ala Leu Val Gly Asn Ser Gly Cys Gly Lys Ser
 1727 ACA ACA CTC CAG CTC ATG CAG AGC CTC TAT GAC CCC ACA GAG 1768
 Thr Thr Val Glu Leu Met Glu Arg Leu Tyr Asp Pro Thr Glu
 1769 GCG ATG CTC AGT GTT GAT GGA CAG GAT ATT AGG ACC ATA AAT 1810
 Gly Met Val Ser Val Asp Gly Glu Asp Ile Arg Thr Ile Asn
 1811 CTA AGC TTT CTA GCG GAA ATC ATT GGT GTC GTC AGT CAG GAA 1852
 Val Arg Phe Leu Arg Glu Ile Ile Gly Val Val Ser Glu Glu
 1853 CCT CTA TTC TTT GGC ACC ACG ATA GCT GAA AAC ATT CGC TAT 1894
 Pro Val Leu Phe Ala Thr Thr Ile Ala Glu Asn Ile Arg Tyr
 1895 GGC CTT GAA AAT CTC ACC ATG GAT GAG ATT GAG AAA GCT GTC 1936
 Gly Arg Glu Asn Val Thr Met Asp Glu Ile Glu Lys Ala Val

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1917 AAG GAA GGC AAT GGC TAT GAC TTT ATC ATG AAA CTG CCT CAT 1970
Lys Glu Ala Asn Ala Tyr Asp Phe Ile Met Lys Leu Pro His

1919 AAA TTT GAC ACC CTG GTT GGA GAG ACA GGG GCT CAG TTG ACT 2020
Lys Phe Asp Thr Leu Val Gly Glu Arg Gly Ala Gln Leu Ser

2021 GGT GAG CAG AAG CAG AUG ATC CTC ATT GGA GGT GCC CTG GTT 2062
Gly Gly Gln Lys Gln Arg Ile Ala Ile Ala Arg Ala Leu Val

2064 GGC AAC GGC AAG ATC CTC CTG CTG GAT GAG GCC ACG TCA GCC 2104
Arg Asn Pro Lys Ile Leu Leu Leu Asp Gln Ala Thr Ser Ala

2105 TTG GAC ACA GAA ACC GAA GCA GTG GTT CAG GTG GCT CTG GAT 2146
Leu Asp Thr Gln Ser Gln Ala Val Val Gln Val Ala Leu Asp

2117 AAG CTC ACA AAA GGT CAG ACC ACC ATT CTC ATA GCT CAT CGT 2188
Lys Ala Arg Lys Gly Arg Thr Thr Ile Val Ile Ala His Arg

2169 TTG TTT ACA GTT GGT AAT GGT GAC CTC ATC GCT CCT TTC CAT 2230
Leu Ser Thr Val Arg Asn Ala Asp Val Ile Ala Gly Phe Asp

2211 CAT GGA CTC ATT CTC GAG AAA GGA AAT CAT GAT GAA CTC ATG 2272
Asp Gly Val Ile Val Gln Lys Gly Asn His Asp Gln Leu Met

2271 AAA GAG AAA GGC ATT TAC TTC AAA GTT CTC ACA ATG CAG ACA 2314
Lys Glu Lys Gly Ile Tyr Phe Lys Leu Val Thr Met Gln Thr

2315 GCA GCA AAT GAA GTT GAA TTA GAA AAT GCA GCT CAT GAA TCC 2356
Ala Gly Asn Gln Val Gln Leu Gln Asn Ala Ala Asp Gln Ser

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2197 AAA AGT GAA ATT GAT GGC TTC GAA ATG TCT TCA AAT GAT TCA 2198
 Lys Ser Glu Ile Asp Ala Leu Glu Met Ser Ser Asn Asp Ser
 2199 AGA TCC AGT CTA ATA ACA AAA AAG TCA ACT CTT AGC ACT CTC 2440
 Arg Ser Ser Leu Ile Arg Lys Arg Ser Thr Arg Arg Ser Val
 2200 GGT GGA TCA GAA GGC CAA GAG AGC AAG CTT AGT ACC AAA GAG 2402
 Arg Gly Ser Glu Ala Glu Asp Arg Lys Leu Ser Thr Lys Glu
 2401 GGT GGT GAT GAA AGT ATA CTT CCA GGT TTC TTT TGG AGC ATT 2524
 Ala Leu Asp Glu Ser Ile Pro Pro Val Ser Phe Trp Arg Ile
 2525 ATG AAG CTA AAT TTA ACT GAA TGG CCT TAT TTT GTT GTT GGT 2566
 Met Lys Leu Asn Leu Thr Glu Trp Pro Tyr Phe Val Val Gly
 2567 CCA TTT TCT GCT ATT ATA AAT GCA GGC CTC CAA CCA GCA TTT 2608
 Val Phe Cys Ala Ile Ile Asn Gly Gly Leu Glu Pro Ala Phe
 2609 GCA ATA ATA TTT TCA AAG ATT ATA GCG GTT TTT ACA AGA ATT 2650
 Ala Ile Ile Phe Ser Lys Ile Ile Gly Val Phe Thr Arg Ile
 2651 GAT GAT GAT GAT GAA ACA AAA CCA CAG AAT ACT AAC TTC TTT TCA 2692
 Asp Asp Pro Glu Thr Lys Arg Glu Asn Ser Asn Leu Phe Ser
 2693 CTA TGG TTT CTA GGC CTT GGA ATT ATT TCT TTT ATT ACA TTT 2714
 Leu Leu Phe Leu Ala Leu Gly Ile Ile Ser Phe Ile Thr Phe
 2715 TTC CTT TAC GGT TTT ACA TTT GAC AAA GGT GCA GAG ATC CTC 2776
 Phe Leu Glu Gly Phe Thr Phe Gly Lys Ala Gly Glu Ile Leu

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2777 ACC AAG CCG CTC CCA TAC ATG GTF TTC CGA TCC ATG CTC AGA 2818
 Thr Lys Arg Leu Arg Tyr Met Val Phe Arg Ser Met Leu Arg
 2819 CAG GAT GTC AGT TCG TTT GAT GAC GCT AAA AAC ACC ACT GGA 2860
 Gln Asp Val Ser Trp Phe Asp Asp Pro Lys Asn Thr Thr Gly
 2861 GCA TTG ACT ACC AGC CTC GGC AAT GAT GCT CAA GTT AAA 2902
 Ala Leu Thr Thr Arg Leu Ala Asn Asp Ala Ala Gln Val Lys
 2903 GCG GAT ATA GGT TCC AGG CTT GCT GTA ATT ACC CAG AAT ATA 2944
 Gly Ala Ile Gly Ser Arg Leu Ala Val Ile Thr Gln Asn Ile
 2945 GCA AAT CTT GCG ACA GGA ATA ATT ATA TCC TTC ATC TAT GGT 2987
 Ala Asn Leu Gly Thr Gly Ile Ile Ile Ser Phe Ile Tyr Gly
 2988 TCG CAA CTA ACA CTC TTA CTC TTA GCA ATT GTA CCC ATC ATT 3028
 Trp Gln Leu Thr Thr Leu Leu Leu Leu Ala Ile Val Pro Ile Ile
 3029 GCA ATA GCA GCA GTT GTT GAA ATG AAA ATG TTC TCT GGA CAA 3070
 Ala Ile Ala Gly Val Val Gln Met Lys Met Leu Ser Gly Gln
 3071 GCA CTC AAA GAT AAG AAA GAA CTA GAA GGT GCT GGG AAG ATC 3112
 Ala Leu Lys Asp Lys Lys Gln Leu Gln Gly Ala Gly Lys Ile
 3113 GGT ACT GAA GCA ATA GAA AAC TTC GGA ACC GTF GTF TCT TTC 3154
 Ala Thr Gln Ala Ile Gln Asn Phe Arg Thr Val Val Ser Leu
 3155 ACT CAG CAG CAG AAG TTT GAA CAT ATG TAT GGT CAG AGT TTC 3196
 Thr Gln Gln Gln Lys Phe Gln His Met Tyr Ala Gln Ser Leu

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1197 CAG GGA GCA TAC AGA AAC TCT TTG AGG AAA GCA CAC ATC TTT
 Gln Val Pro Tyr Arg Asn Ser Leu Arg Lys Ala His Ile Phe 1210

 1209 GGA ATT ACA TTT TTC ACC CAG GCA ATG ATG TAT TTT TCC
 Gly Ile Thr Phe Ser Phe Thr Gln Ala Met Met Tyr Phe Ser 1280

 1281 TAT GGT GGA TGT TTC GGG TTT GCA GGC TAC TTG CTC GCA CAT
 Tyr Ala Gly Cys Phe Arg Phe Gly Ala Tyr Leu Val Ala His 1322

 1321 AAA CTT ATG AGC TTT CAG GAT GGT CTG TTA GTA TTT TCA GCT
 Lys Leu Met Ser Phe Gln Asp Val Leu Leu Val Phe Ser Ala 1364

 1365 GTT CTC TTT GGT GGC ATG GGC CTC GCG CAA CTC AAT TCA TTT
 Val Val Phe Gly Ala Met Ala Val Gly Gln Val Ser Ser Phe 1406

 1407 GCT GGT CAG TAT GGC AAA GGC AAA ATA TCA GCA GGC CAC ATC
 Ala Pro Asp Tyr Ala Lys Ala Lys Ile Ser Ala Ala His Ile 1448

 1449 ATC ATC ATC ATT GAA AAA ACC CTT TTG ATT CAC AGC TAC AGC
 Ile Met Ile Ile Gln Lys Thr Pro Leu Ile Asp Ser Tyr Ser 1490

 1491 ACG GAA GGC CTA ATG CTC AAC ACA TTG GAA GAA AAT GTC ACA
 Thr Gln Gly Leu Met Pro Asn Thr Leu Gln Gly Asn Val Thr 1532

 1531 TTT GGT GAA GTT CTA TTC AAC TAT CCC ACC CCA CCG GAC ATC
 Phe Gly Gln Val Val Phe Asn Tyr Pro Thr Arg Pro Asp Ile 1574

 1575 CCA CTC CTT CAG GCA CTC AGC CTC GAG CTC AAG AGC CCC CAG
 Pro Val Leu Gln Gly Leu Ser Leu Gln Val Lys Lys Gly Gln 1616

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3617 ACC CTG GCT CTG GTC GGC ACC ACT GGC TGT GGC AAG AGC ACA 3658
 Thr Leu Ala Leu Val Gly Ser Ser Gly Cys Gly Lys Ser Thr
 3659 GTC CTC CAG CTC CTG CAG CCG TTC TAC GAC CCC TTG GCA GCG 3700
 Val Val Gln Leu Leu Glu Arg Phe Tyr Asp Pro Leu Ala Gly
 3701 AAA CTC CTG CTT GAT GGC AAA GAA ATA AAG CGA CTC AAT GTT 3742
 Lys Val Leu Leu Asp Gly Lys Glu Ile Lys Arg Leu Asn Val
 3743 CAG TGC CTC CGA GCA CAC CTC GGC ATC GTG TCC CAG GAG CCC 3784
 Gln Trp Leu Arg Ala His Leu Gly Ile Val Ser Gln Glu Pro
 3785 ATC CTG TTT GAC TGC ACC ATT GCT GAG AAC ATT GCC TAT GCA 3826
 Ile Leu Phe Asp Cys Ser Ile Ala Glu Asn Ile Ala Tyr Gly
 3827 CAC AAC ACC CCG CTC CTC TCA CAG CAA GAG ATC GTG AGG GCA 3868
 Asp Asn Ser Arg Val Val Ser Gln Glu Glu Ile Val Arg Ala
 3869 CCA AAG GAG CCC AAC ATA CAT GCC TTC ATC GAG TCA CTG CCT 3910
 Ala Lys Glu Ala Asn Ile His Ala Phe Ile Glu Ser Leu Pro
 3911 AAT AAA TAT ACC ACT AAA GTA GGA GAC AAA GGA ACT CAG CTC 3952
 Asn Lys Tyr Ser Thr Lys Val Gly Asp Lys Gly Thr Gln Leu
 3953 TCT GGT GGC CAG AAA CAA CGC ATT GCC ATA GCT CTT GCC CTT 3994
 Ser Gly Gly Gln Lys Gln Arg Ile Ala Ile Ala Arg Ala Leu
 3995 GTT AGA CAG CCT CAT ATT TTG CTT CTT TTG GAT GAA GCC ACC TCA 4036
 Val Arg Gln Pro His Ile Leu Leu Leu Asp Glu Ala Thr Ser

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4077 GCT CTG CAT ACA GAA AGT GAA ANG GTT GTC CAA GAA GCC CTG 4078
Ala Leu Asp Thr Glu Ser Glu Lys Val Val Gln Glu Ala Leu

4079 GAC AAA GCC AGA GAA GGC CGC ACC TGC ATT GTG ATT GCT CAC 4120
Asp Lys Ala Arg Glu Gly Arg Thr Cys Ile Val Ile Ala His

4121 CGC CTG TCC ACC ATC CAG AAT GCA GAC TTA ATA GTG GTG TTT 4162
Arg Leu Ser Thr Ile Gln Asn Ala Asp Leu Ile Val Val Phe

4163 CAG AAT GGC AGA GTC ANG CAG CAT GCC ACC CAT CAG CAG CTG 4204
Gln Asn Gly Arg Val Lys Glu His Gly Thr His Gln Gln Leu

4205 CTG CCA CAG AAA GGC ATC TAT TTT TCA ATG GTC AGT GTC CAG 4246
Leu Ala Gln Lys Gly Ile Tyr Phe Ser Met Val Ser Val Gln

4247 CCT GUA ACA AAG CGC CAG TGA 4267
Ala Gly Thr Lys Arg Gln Ter

4268 ACTCTGACTG TATCAGATGT TAAATACTTT TTAATATTTG TTTAGATATG
4318 ACATTTTATTC AAGTTAANA CCACACACTT ACAGAAATAT GAAGAGGTAT
4368 CTCTTTANCA TTTCTTCACTT CAACTTLCAG CTTCTCAGAG ACTTCGTAAT
4418 TAAAGGAACA GAGTGAAGA CATCATCAAG TGGAGAGNAA TCATAGTTTA
4468 AACGCAATTA TAAATTTTAT AACAGAAATTA AGTAGATTTT TAAAGATAA
4518 AATGTTTAAAT TTTGTTTATA TTTTCCCATTT TUGACTGTAA CTGACTGCCF
4568 TGTAAAGA TTTATAGAGT ACCAAAAGT ATTGAATGT TTGCATNAAG
4618 TGTCTTAAAT AAACATAAC TTTTCACTGTGA AAAAAAAAAA
4668 AA

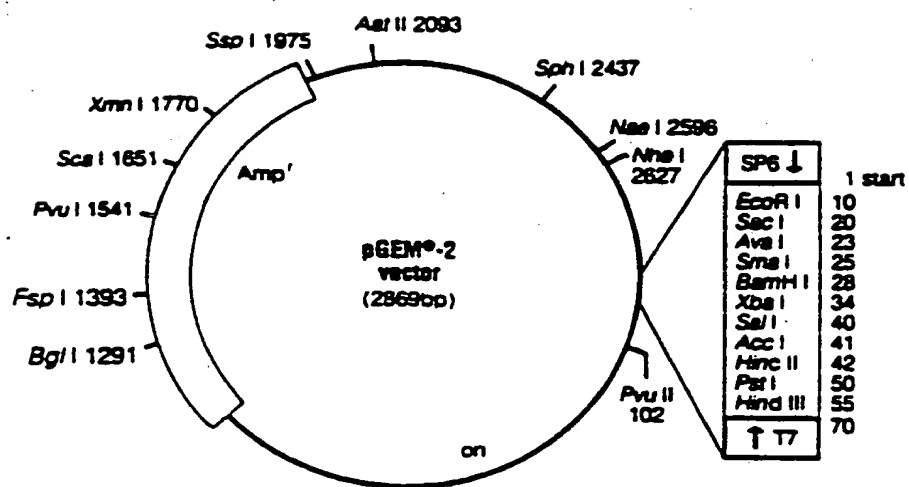


Fig. 5

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Restriction Map of pMDR2000XS (7.3 Kb)

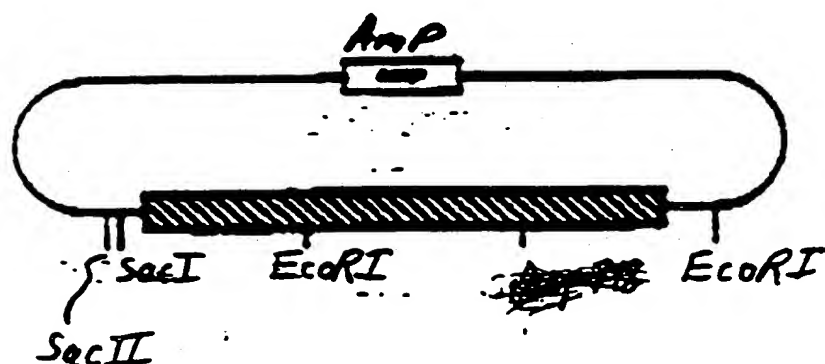


Fig. 6

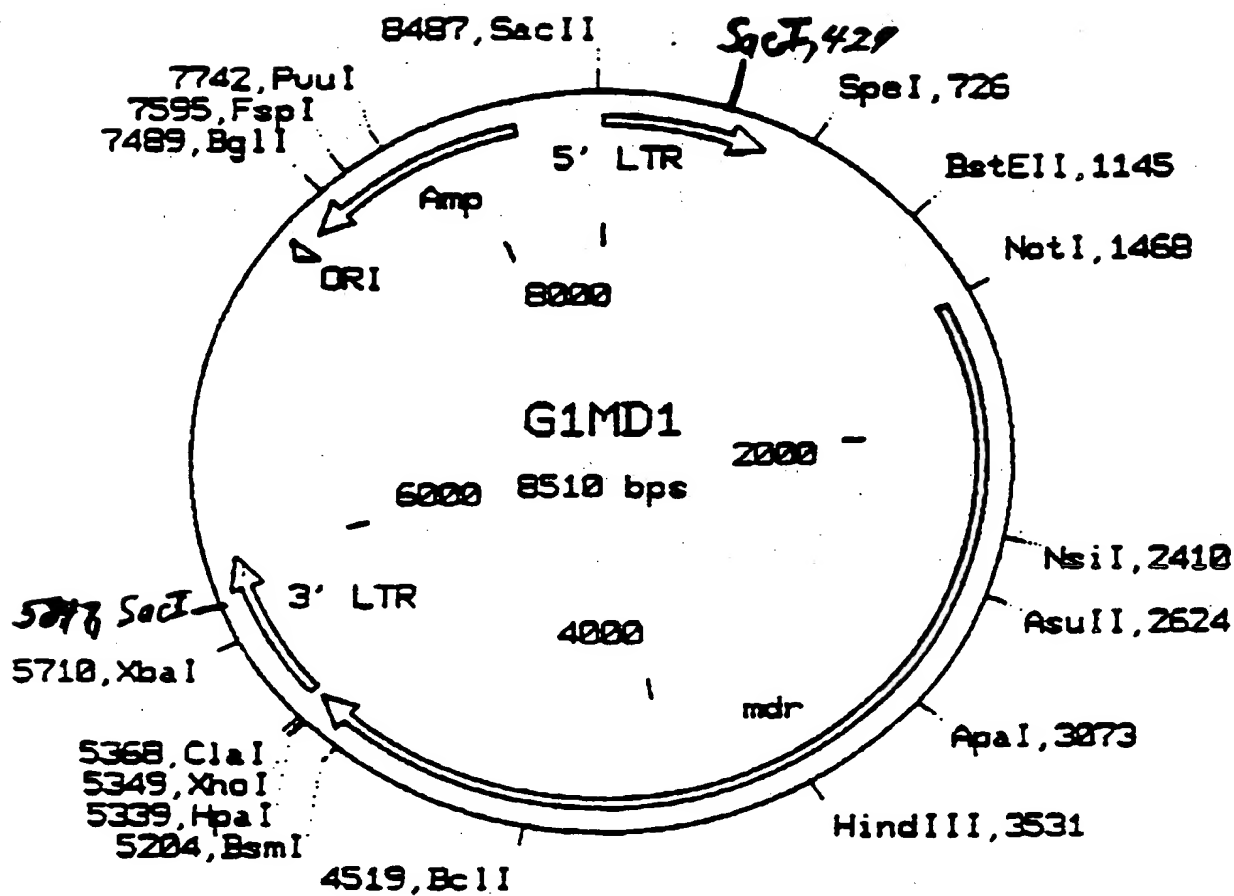
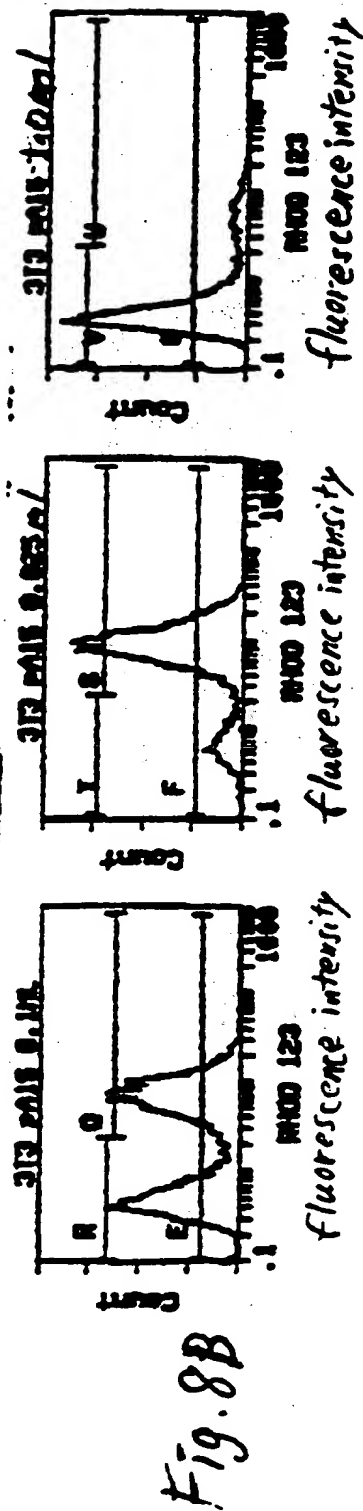
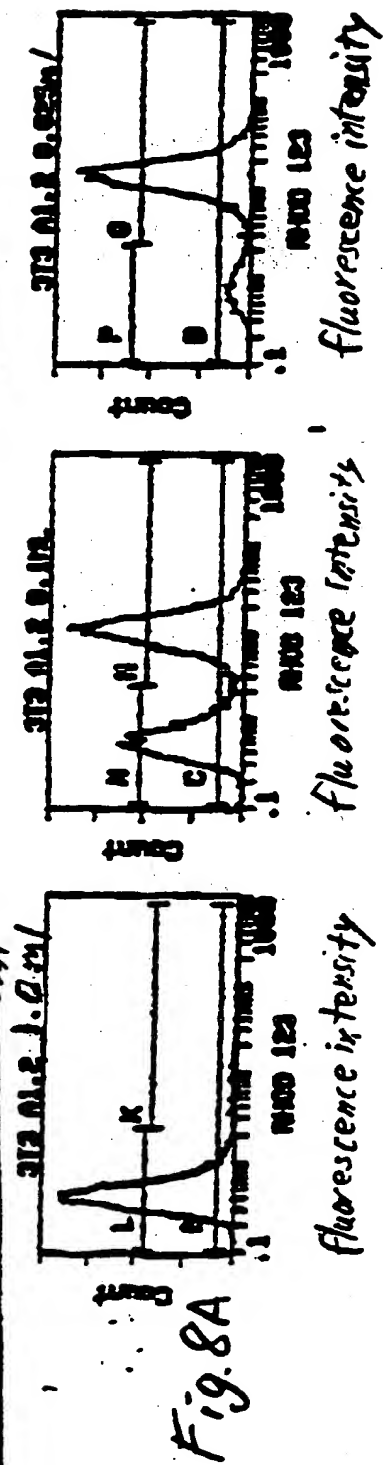


Fig. 7



INTERNATIONAL SEARCH REPORT

International application No.

PCT/US93/04707

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) : C12N 5/10, 15/12; G01N 33/52

US CL : 536/23.5; 435/6, 69.1, 320.1, 240.2

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 536/23.5; 435/6, 69.1, 320.1, 240.2

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, DIALOG, SwissProt, EMBL, GENBANK, PIR, search terms: multidrug, resistance, slice, donor, acceptor, sequence, consensus

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
<u>X</u> Y	The Journal of Biological Chemistry, Volume 266, Number 7, issued 05 March 1991, Devine <i>et al.</i> , "Full length and alternatively spliced pgp ₁ transcripts in multidrug-resistant Chinese hamster lung cells", pages 4545-4555, see especially Figure 7 and column 1 of page 4555.	<u>12, 14, 16</u> 1-24, 27, 28
<u>X</u> Y	Cell, Volume 66, issued 12 July 1991, Chaudhary <i>et al.</i> , "Expression and activity of P-glycoprotein, a multidrug efflux pump, in human hematopoietic stem cells", pages 85-94, see especially page 85.	<u>27-31</u> 24-26
Y	Journal of Molecular Biology, Volume 195, issued 1987, Ohshima <i>et al.</i> , "Signals for the selection of a splice site in pre-mRNA; computer analysis of splice junction sequences and like sequences", pages 247-259, see especially page Figures 1 and 2 and page 248.	1-24, 27, 28



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents:	*T	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
A document defining the general state of the art which is not considered to be part of particular relevance	*X*	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
E earlier document published on or after the international filing date	*Y*	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
L document which may throw doubt on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*Z*	document member of the same patent family
O document referring to an oral disclosure, use, exhibition or other means		
P document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search

17 August 1993

Date of mailing of the international search report

25 AUG 1993

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US93/04707

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Cell, Volume 47, issued 21 November 1986, Aebi <u>et al.</u> , "Sequence requirements for splicing of higher eukaryotic nuclear pre-mRNA", pages 555-565, see especially Figure 9 and page 562.	1-24, 27, 28
Y,P	US, A, 5,175,099 (Wills) 29 December 1992, see entire document, especially column 6.	1-24, 27, 28
Y,P	US, A, 5,206,352 (Roninson <u>et al.</u>) 27 April 1993, see Figure 5 and columns 1 and 2.	1-24, 27, 28-31
Y,P	US, A, 5,192,553, (Boyse <u>et al.</u>) 09 March 1993, see the abstract and columns 7 and 8.	27-31

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